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By:_____
Karen A. Herrand

Docket No.: 0656-008US6

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	Skiffington et al.	Examiner:	Beisner, William H.
Reissue Serial No:	10/014,154	Art Unit:	1744
Filed:	December 6, 2001		
Original Patent No:	6,180,395		
Original Patent Issue Date:	January 30, 2001		
Title:	Reagent Chamber for Test Apparatus and Test Apparatus		

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APPEAL BRIEF

This is an appeal from the final rejection of claims 1, 2, 5-7, 10, 12, 14, 15, 17-19, 23, 24 and 26 of the Office Action dated January 31, 2006. Appellant submits this Appeal Brief pursuant to 35 U.S.C. §134 and 37 CFR §41.37, further to the Notice of Appeal filed on April 12, 2006. A petition for extension of time and the required fees are enclosed as indicated in the accompanying Transmittal and Fee Transmittal forms.

I. Real Party in Interest

The real party in interest is Charm Sciences, Inc., the assignee of record, which is a corporation of the Commonwealth of Massachusetts having a principle place of business at 659 Andover Street, Lawrence, MA 01843.

II. Related Appeals And Interferences

There are no appeals or interferences related to the present appeal.

III. Status of Claims

Claims 1, 2, 4, 5-7, 10, 12, 14, 15, 17-19, 23, 24, 26, and 30 are pending in the application. Claims 4 and 30 are allowed. Claims 3, 8, 9, 11, 13, 16, 20-22, 25, 27-29, and 31-45 are cancelled. Claims 1, 2, 5-7, 10, 12, 14, 15, 17-19, 23, 24 and 26 stand rejected and are involved in this appeal.

IV. Status of Amendments

There have been no amendments filed after the final rejection of January 31, 2006.

V. Summary of Claimed Subject Matter

The claims on appeal are each directed to a single use test apparatus device for the luminescent detection of ATP in a test sample, or to particular portions thereof. Claims 5-7, 10, 14, 15, and 17-19 are directed to an ATP detection test apparatus containing one or more unit dose reagent chambers, claims 23 and 26 are directed to embodiments of the test unit portion of the test apparatus in which unit dose reagent chambers are assembled as part of the test unit, and claims 1, 2, and 12 are directed to unit dose reagent chambers particularly adapted to use in an ATP detection apparatus. A more particular summary of the claims is set forth below.

By way of overview, the invention relates to the field of hygiene monitoring, by providing a rapid, portable, and inexpensive test for luminescent detection of ATP in test samples taken from various materials or surfaces with the use of a swab. Original Patent, col. 1, lines 30-39. The bioluminescent ATP detection assay is conducted in a homogenous liquid reaction medium. The test sample is suspended in a solution containing an ATP releasing reagent composition, and ATP content is determined by measuring the luminescence produced in a luciferase-catalyzed reaction between the substrate luciferin and any ATP in the sample.

All reagents are housed, and all steps of the reaction are performed, within the test apparatus device, so the device must be able sequester prepackaged liquid reagents within the same device that also houses reagents that are optimally kept dry to retain stability. An ATP detection apparatus must be suitable for performing a homogeneous chemical reaction in a liquid medium, and for measuring rapid luminescent output as an indication of ATP content in the test sample.

The test apparatus of the invention meets these needs by generally including an elongated housing portion, a test unit portion, one or more unit dose reagent chambers containing ATP detection specific reagents, and a moveable probe which obtains sample, releases reagent from one or more reagent chambers, and aids the admixture of sample and reagents in the bottom end of the test unit. Luminescence resulting from reaction of luciferin with any ATP in the test sample is observed directly through the walls of the test unit, which are preferably transparent so that a test results can be observed with a luminometer. The test unit can be an integral portion of the test apparatus, in which case the bottom test unit portion of the test apparatus is placed directly into the luminometer. Alternatively, the test unit may be detached for use in a test instrument, or to conduct tests on the admixture therein.

Generally, the reagent chamber is characterized by a package having a puncturable foil seal or membrane, which is adapted to be penetrated by the movement of the probe after collection of the test sample by the probe. Generally the reagents are also packaged and separated in order to provide for better storage life. As the test sample on the probe is pushed through the reagent chambers, it comes in contact with each of the selected reagents, with the test reagents and test samples admixed at the bottom end of the housing of the test unit. One of the test reagents, either liquid or dry, may also be placed in the bottom end of the test unit to be admixed with the probe-released reagents and test sample.

Claims 1, 2, and 12

The unit dose reagent chamber of claims 1, 2, and 12 contains an adenosine triphosphate (ATP) detection reagent and is designed to be suitable for assembly into an ATP detection test apparatus equipped with a moveable probe that is used to obtain the test sample and to release the ATP detection reagent from the reagent chamber into a test unit. The reagent chamber is cylindrical with a probe-puncturable membrane seal over the one end and the other end of the cylinder to provide a sealed compartment which encases the ATP detection reagent. The ATP detection reagent within the reagent chamber is specifically selected from a detergent-containing buffered solution to

release adenosine triphosphate (ATP) from the test sample into the solution for testing, and a luciferin-luciferase reagent. The membrane seal can be prepared using an aluminum foil **(claim 2)**. The ATP detection reagent sealed within the chamber can be chosen to be a biological buffer solution which optimizes the conditions of the ATP detection reaction **(claim 12)**. (*See, e.g.,* the specification of the original patent at col. 4, lines 27-36, col. 7 lines 24-25 and lines 56-60, col. 8 line 65 to col. 9 line 2, col. 10 line 65 to col. 11 lines 1-3, col. 11 lines 11-18, col. 12-13 example 1, and Fig. 6.)

Claims 5, 6, 7, and 10

Claim 5 is directed to a combination device resulting from assembly of one or more of the unit dose reagent chambers of claim 1 into the ATP detection test apparatus for which it was designed. As a dependent claim, claim 5 incorporates all of the limitations of claim 1, and so the test apparatus of claim 5 must be suitable for conducting an ATP detection reaction in a test sample, and must also employ a moveable probe to obtain a test sample and to release one or more reagents from within a unit dose reagent chamber to a test unit. In certain embodiments, the moveable probe moves longitudinally within the test apparatus, so that the longitudinal force of the probe's movement punctures the membrane seals of the unit dose reagent chamber(s) **(claim 6)**. Claim 5 further requires that the unit dose reagent chamber portion of the test apparatus contain a detergent-containing buffered solution for releasing ATP into the solution for testing, and that a luciferin-luciferase reagent be present elsewhere within the test apparatus, such as, e.g., in the test unit portion of the test apparatus **(claim 10)**. Preferably, the test unit is transparent and has a closed bottom end **(claim 7)**. The embodiment of claim 7 positions one or more unit dose reagent chambers in the test unit portion of the test apparatus. (*See, e.g.,* col. 3, lines 5-13 and col. 3, line 40-55.)

Claims 14, 15, and 17-19

Claim 14 is directed to a test apparatus designed for use in detecting ATP in a test sample by luminescence. The test apparatus has a longitudinal housing. A test unit portion extends from one end of the housing. The test unit features a closed bottom end, in which an ATP reagent is stored and in which the ATP detection reaction takes place. The test unit portion is transparent, so that the results of the luciferase reaction test can be observed through the walls of the test unit by chemiluminescence. The unit dose reagent chambers are longitudinally-positioned in the test unit portion of the test apparatus. The test unit portion can be an integral portion of the test apparatus.

Alternatively, the test unit is detachably secured to the one end of the test apparatus, permitting the test unit to be detached from the remaining portions of the test apparatus **(claim 17)**. The membrane seal can be prepared using aluminum foil **(claim 15)**. (*See, e.g.,* col. 6 lines 39-40, col. 10 lines 8-9, and col. 10, line 9.)

Claims 23 and 26

Claim 23 is directed to a transparent test unit for use in a test apparatus for the detection of a test sample. The test unit has a closed bottom end, a probe-puncturable membrane over the other end, one or more unit dose reagent chambers longitudinally positioned in the test unit, and means for detachably securing the test unit to a test apparatus. (*See, e.g.,* col. 3 lines 46-50, col. 6 lines 25-67, col. 7 lines 1-10, col. 8 lines 28-44, col. 10 lines 32-62, col. 11 lines 20-29 and 60-67, and Figs. 3, 4, 5, 7, and 8.)

VI. Grounds of Rejection To Be Reviewed On Appeal

1. Whether claims 1, 2, 5-7, 10 and 12 are unpatentable under 35 U.S.C. § 103(a) over Bernstein (US 4,770,853; "Bernstein") in view of Simpson et al. (EP 0 309 184; "Simpson et al.") and Rich et al. (US 3,666,631; "Rich et al.").

2. Whether claims 10, 14, 15, 17-19, 23, 24 and 26 are unpatentable under 35 U.S.C. § 103(a) over Bernstein (US 4,770,853) in view of Simpson et al. (EP 0 309 184; "Simpson et al.") and Rich et al. (US 3,666,631; "Rich et al.") and taken further in view of Matsumoto et al. (JP 7-59555).

VII. Argument

I. The examiner's rejection of claims 1, 2, 5-7, 10 and 12 under 35 U.S.C. § 103(a) as not being patentable over Bernstein (US 4,770,853; "Bernstein") in view of Simpson et al. (EP 0 309 184; "Simpson") and Rich et al. (US 3,666,631; "Rich") should be reversed.

Claims 1, 2, 5-7, 10 and 12 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Bernstein (US 4,770,853; "Bernstein") in view of Simpson et al. (EP 0 309 184; "Simpson et al.") and Rich et al. (US 3,666,631; "Rich et al."). Applicant respectfully requests that the rejection be reversed.

The examiner must establish factual basis for obviousness to a preponderance of the evidence, by determining the scope and content of the prior art, identifying the differences between

the prior art and the claimed invention as a whole, determining the level of skill in the art, and providing factual support for finding a greater than 50% likelihood that one of ordinary skill in the art would not merely have been motivated to solve the problem, but be motivated to arrive at the same solution as that claimed. Facts established by rebuttal evidence must be evaluated along with the facts on which the conclusion of a prima facie case was reached, not against the conclusion itself. In other words, each piece of rebuttal evidence should not be evaluated for its ability to knockdown the prima facie case. All of the competent rebuttal evidence taken as a whole should be weighed against the evidence supporting the prima facie case. *In re Piasecki*, 745 F.2d 1468, 1472, 223 USPQ 785, 788 (Fed. Cir. 1984); MPEP 716.01(d).

The examiner has not met that burden. First, the examiner has not taken all claim limitations into account, and thus has not considered the invention as a whole. Second, as demonstrated by the Second Declaration of Steven J. Saul (Exhibit A) and the Childs et al. patent (Exhibit B), it has not been established to a preponderance of the evidence that the skilled artisan would find a suggestion or motivation in the references to modify the Bernstein apparatus according to the disclosures of Simpson and Rich. As testified to by Dr. Saul, one of ordinary skill in the art in 1995, reading the Bernstein, Simpson, and Rich patents, would not have been motivated to provide a unit dose reagent chamber containing a luciferin-luciferase reagent by placing luciferin-luciferase in the vessel of the Bernstein apparatus, because the Bernstein apparatus is not suitable for chemiluminescent detection of ATP, and because modification of the Bernstein apparatus for chemiluminescent detection of ATP would have made the Bernstein apparatus unsuitable for its intended purpose of a solid phase immunodiffusion assay. Dr. Saul further testifies that one of ordinary skill in the art in 1995, reading the Bernstein, Simpson, and Rich patents, would not have been motivated to provide a unit dose reagent chamber containing a detergent-containing buffered solution for use in a test apparatus for detecting ATP in a test sample, by placing a detergent-containing buffered solution in the vessel of the Bernstein apparatus. The Bernstein apparatus features an open portal window and relies for its operation on the presence of a prefilter and capture membrane, and thus would not be suitable for use in ATP detection. Modification of the Bernstein apparatus to be suitable for ATP detection, by the substantial reconstruction of eliminating the prefilter and capture membrane, would result in leakage of unabsorbed fluid out of the window.

Finally, the Childs patent is relevant as a secondary consideration of non-obviousness. *Monarch Knitting Machinery Corp. v. Sulzer Morat GmbH*, 139 F.3d 877, 45 U.S.P.Q.2d 1977,

1983 (Fed.Cir. 1998) (*"Therefore, this court will address the evidence of contemporaneous invention in that context."*). The Childs patent demonstrates that others of ordinary skill in the art in 1995, seeking to provide a device for chemiluminescent detection of detergent-released ATP in a test sample and in similar possession of Bernstein, Simpson, and Rich, were *not* motivated to provide a unit dose reagent chamber for ATP detection containing a detergent-containing buffered solution or a luciferin-luciferase reagent. Childs et al. arrived at a completely different solution than that of the claimed invention. Evidence in the form of the Childs patent reduces the likelihood that one skilled in the art would be motivated by the references to arrive at the claimed invention to below 50%. The rejection can not be maintained.

A. <u>The Examiner's Rejection</u>

The examiner states his case of obviousness as follows:

> In view of these teachings, it would have been obvious to one of ordinary skill in the art at the time the invention was made to provide the adenosine triphosphate detection reagents as taught by the prior art references of Simpson et al. and Rich et al. within the test device structure as disclosed by the reference of Bernstein for the known and expected result of employing an alternative means recognized in the art for storing and performing a multiple step assay while providing the benefits disclosed by the reference of Bernstein when using the disclosed reagent holding system (See column 1, lines 4-28).

<u>Office Action</u>, January 31, 2006, page 5, lines 4-10. Bernstein summarizes the nature of the Bernstein "device" as follows:

> [A] device for a self contained solid phase immunodiffusion assay. The device is comprised of a sample collector, a tube with compartmentalized reagents and a ligand receptor capture membrane filter area. The sample collector is pushed through the seals, mixed with reagent, and then pushed into a ligand receptor reaction area wherein the tip of the sample collector contacts diffusible membranes or filters and transfers the reactants to a capture membrane wherein a ligand receptor reaction an be visualized by the naked eye.

<u>Bernstein</u>, abstract. The examiner has acknowledged that the Bernstein reference

> "does not disclose the use of reagents specific for the detection of adenosine triphosphate wherein the reagent is either a detergent-containing buffered solution to release adenosine triphosphate from a test sample or a luciferin-luciferase reagent."

Office Action, January 31, 2006, page 4, ¶ 3. Beyond that, the examiner's remaining findings of fact bear closer scrutiny.

1. The examiner misquotes Bernstein and is misleading when stating that,

> While the preferred embodiment of the reference of Bernstein is directed to the performance of an immunoassay detection, the reference discloses that the device is advantageous for assays that require multiple steps and require multiple reagents (See column 1, lines 13-28).

Office Action, January 31, 2006, page 4, lines 1-4 of ¶ 4. A review of the cited passage of Bernstein reveals that Bernstein makes no such statement about the Bernstein device. The cited passage is the first paragraph of Bernstein's background section, in which Bernstein articulates the problems of bringing diagnostic assay procedures

> to physicians offices and even to the home, where untrained or poorly trained individuals perform the tests usually following product insert instructions alone. These assays are useful provided they are performed properly and are safe handle for the user. Assays that require multiple steps, have multiple reagents, and have limited storage conditions are prone to misuse, especially if they are performed by individuals without adequate training or skills.

Bernstein, col. 1, lines 17-28. Read in context, the cited passage is merely an observation as to difficulties faced in the field of point-of-care diagnostics. The passage does not make claims specific to the Bernstein device.

2. The examiner continues with the statement,

> The reference also discloses a number of types of reagents that can be used in the device including extraction reagent and lyophilized reagents (See column 3, lines 11-28).

Office Action, page 4, lines 4-5 of ¶ 4. The passage of Bernstein at column 3, lines 11-28, is a general list of reagents frequently used in the context of ligand-receptor assays. The list omits any specific suggestion of reagents useful for chemiluminescent detection of ATP. Such a general teaching is valuable only when interpreted through the filter of one of ordinary skill in the art having in mind the development of a particular type of assay. In the Second Declaration of Steven J. Saul, Dr. Saul testifies as to how one skilled in the art of single-use ATP detection test devices would have interpreted the disclosure of Bernstein.

3. Simpson et al. is directed to a procedure for extracting ATP using a detergent-containing buffered solution to release ATP into a test solution. The procedure involves extracting ATP from a suspension of the microorganism with a cationic surface active agent (detergent), then neutralizing the cationic surface active agent. Simpson, page 4, lines 21-25. The assay of Simpson et al. is

performed by pipetting ATP extraction reagents, sample, and luciferase-luciferin reagents into a standard laboratory cuvette and reading luminescent output with a luminescent photometer.

4. In Rich, a pressure roller pushes sample into a compressible plastic bag-type chamber 78 containing liquid ATP extractants. After a five minute incubation, the pressure roller compresses the chamber 78, forcing the fluid through an orifice 82 into a second plastic bag-type chamber 80, which contains luciferin-luciferase reagents. The light emitted from the luciferin/luciferase reaction is then measured by photographic exposure through a window onto Polaroid film.

B. Applicant's Rebuttal Evidence

Evidence traversing rejections, when timely presented, must be considered by the examiner whenever present. MPEP § 716.01(B). Where, as here, the examiner has determined that the evidence is insufficient to overcome the rejection, the examiner must specifically explain why the evidence is insufficient. Id. General statements such as "the declaration is devoid of any factual evidence" are insufficient. *See,* id. Here, applicant timely submitted two pieces of evidence in rebuttal of the examiner's obviousness determination: the Second Saul Declaration (Exhibit A), and the Childs et al., U.S. 5,783,399, another patent on which David Bernstein a named inventor (Exhibit B).

The Second Saul Declaration provides testimony as to the understanding of one of ordinary skill in the art, and should be given greater weight.

The Second Saul Declaration was submitted as rebuttal evidence in response to the non-final office action issued March 28, 2005. Although the record reflects that the declaration was entered, the examiner did not give its testimony any weight. The examiner reasoned that,

> In this case, the opinion evidence has been submitted to convey on the record that one of ordinary skill in the art would not have been motivated to modify the Bernstein apparatus for chemiluminescent detection of ATP because the modification would have made the apparatus of Bernstein unsuitable for its intended purpose of a solid phase immunodiffusion assay; the declarant is an employee of the assignee of the instant application and thus has an interest in the outcome of the application; and the declaration is devoid of any factual evidence supporting the statements within the declaration.

Final Office Action, January 31, 2006, paragraph bridging pages 8-9. For all of the reasons set forth below, applicant respectfully requests that the Board grant *de novo* review of the testimony set forth in the Second Saul Declaration.

Dr. Saul is not a party having an interest in the outcome of the application.

The examiner relies on *In re Lindell* as a basis for finding that Dr. Saul has an interest in the outcome of the application. Final Office Action, January 31, 2006, page ; It is not clear how the facts of *In re Lindell* pertain to the instant declarant. In *In re Lindell,* the Court of Customs and Patent Appeals (CCPA) gave little credit to the affiant's testimony because the testimony was that of the patent applicant himself. The facts of this case do not rise to that level. As shown by all of the application transmittal papers of record, the oath and declaration of the original patent, and each of the supplemental oaths and declarations submitted in the current reissue application, the named inventors of the instant application have declared under oath that Dr. Steven J. Saul is not an inventor of any of the subject matter on appeal. Nor is Dr. Saul an assignee or any other party that would qualify him as a real party in interest. As an employee of the assignee Dr. Saul is admittedly not fully at arms length, but his interests are decidedly minor when considered in the light of the facts of *In re Lindell.* Dr. Saul has given his testimony under oath and penalty of perjury. Exhibit A, ¶ 23. His relatively minor interests as a salaried employee should not be permitted to over-ride the probative value of his testimony.

Dr. Saul's testimony as to how one of skill in the art would interpret the Bernstein reference has probative value as to the factual underpinnings required to support a conclusion of obviousness.

The examiner erred in viewing the Second Saul Declaration as being no more than "declarant's opinion on the ultimate legal issue". Final Office Action, January 31, 2006, paragraph bridging page 8-9 (*citing, In re Lindell,* 155 USPQ 521 (CCPA 1967). The Second Saul Declaration offered factual evidence in an attempt to explain how one of ordinary skill in the art would have understood the Bernstein reference. The Second Saul Declaration should be considered, not as probative of the ultimate legal conclusion of obviousness, but for its testimony as to the factual underpinnings of that conclusion. *In re Alton*, 76 F.3d 1168, 1174-75, 37 USPQ2d 1578, 1582-83 (Fed. Cir. 1996) (*patent examiner erred by dismissing declaration of applicant's expert without adequately explaining how declaration failed to overcome prima facie case supporting rejection; examiner did not address expert's argument that one of ordinary skill in the art would have understood specification"*).

The examiner further erred when finding that "the declaration is devoid of any factual evidence supporting the statements within the declaration." *Id.* Such a finding is inappropriately hasty and dismissive. In point of fact, Dr. Saul, as an expert in the field, is qualified to testify as to the views of one of ordinary skill in the art as of the benefit date accorded the instant application. Exhibit A ¶¶1-3; Exhibit A1; Exhibit C ¶¶1,2,4; Exhibit C1. Dr. Saul is therefore qualified to testify as to how one of ordinary skill in the art would have understood the Bernstein reference. In particular, Dr. Saul has set forth the following factual evidence:

1. Dr. Saul testified that the Bernstein apparatus features an open portal window and relies for its operation on the presence of a prefilter and capture membrane, which would not be suitable for use in ATP detection. Modification of the Bernstein apparatus to be suitable for ATP detection would require the substantial reconstruction of eliminating the prefilter and capture membrane, and would result in leakage of unabsorbed fluid out of the window. Exhibit A, ¶ 10.

2. Dr. Saul testified that, based on his review of the Bernstein patent, it is his view that Bernstein sought to provide a test device suitable for performing a ligand receptor assay to detect antigens, haptens, antibodies, DNA or RNA fragment, wherein the user is not required to dispense any of the reagents. Further design criteria were that all reagents be self-contained within a device that could be stored at nonrefrigerated temperatures, and which could utilize lyophilized reagents. Exhibit A, ¶ 11, citing, Bernstein, col. 2, line 56, to col. 3, line 2.

3. Referring to col. 2, lines 46-55, Dr. Saul further testified that Bernstein states that it is an object to transfer the reactants "to a reaction zone where the specific labeled reactant can be captured and visualized." Dr. Saul further stated that, at col. 1, paragraph 2, the Bernstein patent expresses the goal of eliminating any need for capital equipment such as "scintillation counters, flourometers and colorimeters in the case of radioimmunoassay, fluorescent immunoassay, and enzyme immunoassay respectively". Exhibit A, ¶ 12 (citing Bernstein, col. 2, lines 46-55).

4. It is the opinion of Dr. Saul that the Bernstein apparatus is constructed so as to accomplish the goal of performing a rapid solid phase immunodiffusion assay, at col. 3, lines 49-52, Bernstein states that "[t]he configuration of the lower portion allows the collection device to come into physical contact with the prefilter, capture membrane or capture filter." Exhibit A, ¶ 13.

5. Dr. Saul testifies that Bernstein states that "[i]n the case where membranes or filters are used to capture the immunoreactants, it is necessary to bring the fluid containing the immunoreactants in contact with the filter or membrane." Exhibit A, ¶ 14 (*citing,* Bernstein, col. 2, lines 26-29).

6. Dr. Saul observes that Bernstein further articulates the importance of having a larger pore size filter or membrane between the swab and capture membrane to retain any unwanted cells or debris that may interfere with the assay. Exhibit A, ¶ 15 (*citing,* Bernstein, col. 2, lines 46-55).

7. Dr. Saul testified that the Bernstein apparatus is also configured so that the assay results can be observed visually through a window, which is a discrete observation portal on the front side of the lower portion of the device. Exhibit A, ¶ 16.

8. In order to visualize the signal without the aid of capital equipment, it was necessary to concentrate the signal in front of the window. Exhibit A, ¶ 16.

9. Dr. Saul testified that, to concentrate the signal in front of the window Bernstein had to do four things: (a) capture the labeled members of the binding pair on capture membranes 18, 19; (b) eliminate interfering substances on a pre-filter membrane 25; (c) remove excess fluid on absorbent 17; and (d) deliver the reagents into direct proximity in front of the prefilter and reaction membranes. Bernstein, col. 3, lines 34-51. Dr. Saul further testified that, at col. 5, lines 5-8, Bernstein states, "The shape of the lower portion 10 is configured to enhance contact of the collection device tip with the pre-filter or reaction membranes." Absent each of these design features, Bernstein would not be able to achieve sufficient signal enhancement for visualization through the front window 11. Exhibit A, ¶ 17.

10. It is the opinion of Dr. Saul that it would not have been obvious to one of ordinary skill in the art in 1995 to modify the Bernstein apparatus to be suitable for chemiluminescent detection of ATP with luciferin-luciferase. Adaptation of the Bernstein apparatus for chemiluminescent detection would have required modification of the device to be suitable for use with a luminometer. Those skilled in the art would not have found a suggestion or motivation to modify the Bernstein apparatus for use with a luminometer. To do so would have contradicted Bernstein's goal of providing a rapid immunodiagnostic assay that operated independently of capital equipment. Exhibit A, ¶ 18.

11. Dr. Saul further testified that another reason those skilled in the art would not have adopted the Bernstein apparatus for use with a luminometer is that, were one to do so, the Bernstein apparatus would have become inoperable for its intended purpose of visualization of signal by the naked eye. The shape of the lower portion of the Bernstein apparatus is configured to enhance contact of the collection device tip with the prefilter or reaction membranes. Were the lower portion of the Bernstein apparatus to be modified to fit inside a luminometer, its shape would no-longer be

configured to enhance contact of the collection tip with the prefilter or reaction membranes. Exhibit A, ¶ 19 (citing Bernstein, col. 5, lines 5-7).

12. Dr. Saul further testified that one skilled in the art would not have been motivated to modify the Bernstein apparatus to operate without prefilter or reaction membranes. Were the Bernstein apparatus to have been so adapted, there would be no concentration of signal in front of window 11, and the Bernstein apparatus would then be unsatisfactory for its intended purpose. Exhibit A, ¶ 20.

13. Dr. Saul further testified to his opinion that one skilled in the art in 1995 would not have been motivated to place a detergent-containing buffered solution into the vessel of the Bernstein device, because the Bernstein apparatus does not contain a closed bottom end. Dr. Saul observed that Bernstein describes, at col. 5, lines 15-25, an "adhesive tape 12 that holds the absorbent [17] in place and applies the necessary pressure to ensure diffusion of fluid through the various layers of the ligand receptor test area." The absorbent 17 absorbs excess fluid diffusing through the membranes. By removing adhesive tape 12 by lifting tab 28 of Bernstein, the bottom end of the Bernstein device is not a closed bottom end. Exhibit A, ¶ 21 (*citing*, Bernstein, col. 5, lines 15-25).

14. It is Dr. Saul's opinion that one skilled in the art would not be motivated to undergo the substantial reconstruction of the Bernstein device that would be required to make it suitable for detecting ATP using a detergent-containing buffered solution. The bottom of the Bernstein device is not closed, so any solution would leak out the "window." Exhibit A, ¶ 22.

C. Taken as a whole, the evidence of record does not support a prima facie case of obviousness

When identifying differences between the claimed invention and the teachings of the prior art, all words in applicant's claim must be considered in judging the patentability of that claim against the prior art. MPEP 2143.03 (citing *In re Wilson*, 424 F2d 1382, 1385, 165 USPQ 494, 496 (CCPA 1970)(reversing Board's finding of obviousness where Board ignored language in the claim).

Claims 5-7 and 10

As a combination claim, claim 5 is directed to an ATP detection test apparatus containing one or more of the unit dose reagent chambers of claim 1. As a dependent claim, claim 5 incorporates all of the limitations of claim 1, and so the test apparatus of claim 5 must be suitable for conducting an ATP detection reaction in a test sample, and must also employ a moveable probe to obtain a test sample and to release one or more reagents from within a unit dose reagent chamber to

a test unit. In certain embodiments, the moveable probe moves longitudinally within the test apparatus, so that the longitudinal force of the probe's movement punctures the membrane seals of the unit dose reagent chamber(s) **(claim 6)**. Claim 5 further requires that the unit dose reagent chamber portion of the test apparatus contain a detergent-containing buffered solution for releasing ATP into the solution for testing, and that a luciferin-luciferase reagent be present elsewhere within the test apparatus, such as, e.g., in the test unit portion of the test apparatus **(claim 10)**. Preferably, the test unit is transparent and has a closed bottom end **(claim 7)**. The embodiment of claim 7 positions one or more unit dose reagent chambers in the test unit portion of the test apparatus.

Critical features of the Bernstein test apparatus make it decidedly unsuitable for chemiluminescent detection of ATP, and unsuitable for use in an ATP detection reaction requiring a detergent-containing buffered solution. Although the examiner acknowledges that the Bernstein test apparatus has been designed to perform an immunoassay, not to detect ATP, the examiner asserts that col. 1, lines 13-28, and col. 3, lines 11-28 support a broader interpretation of Bernstein. Office Action, January 31, 2006, page 4. As discussed above, the cited passages are inconsistent with that assertion. The list omits any specific suggestion of reagents useful for chemiluminescent detection of ATP. Neither of these general statements broaden the scope of suitability of Bernstein's test apparatus to chemiluminescent detection of detergent-released ATP. By the examiner's reasoning, one skilled in the art would have been motivated to modify the Bernstein apparatus for any multi-step assay by placing ***any*** reagents into the vessels of Bernstein. In taking this position, the examiner does not address the specific language of the claim 1. Focusing on the obviousness of substitutions and differences, instead of on the invention as a whole, is a legally improper way to simplify the question of obviousness. *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 231 USPQ 81, 93 (Fed.Cir. 1986).

There is no suggestion or motivation to combine the cited references

The rejection should be withdrawn as lacking any rationale as to how or what would have suggested or motivated the skilled artisan to modify the Bernstein apparatus to provide the claimed invention. Merely alleging that the references can be combined or modified does not make the resulting combination obvious unless the prior art also suggests the desirability of the combination. MPEP 2143.01.

There is no motivation to combine references where, as here, the proposed modification would change the principle of operation of the prior art apparatus of the primary reference, and would render the prior art apparatus unsatisfactory for its intended purpose.

It is well established that no suggestion or motivation to combine is present where the proposed modification would change the principle of operation of the prior art reference. MPEP 2143.01 (p. 2100-132), *citing, In re Ratti*, 270 F.2d 810, 813, 123 USPQ 349, 352 (CCPA 1959) (*obviousness rejection reversed where suggested combination of references would require a substantial reconstruction and redesign of the elements shown in the primary reference as well as a change in the basic principle under which the primary reference construction was designed to operate*). It is also well established that there is no suggestion or motivation to combine the references where the proposed modification would render the prior art unsatisfactory for its intended purpose. MPEP 2143.01 (p. 2100-131), *citing, In re Gordon,* 733 F.2d 900, 221 USPQ 1124 (Fed. Cir. 1984) (BPAI's conclusion of *prima facie* obviousness reversed based on finding that, were prior art device to be turned upside down, it would have been inoperable for its intended purpose).

Bernstein is directed to an apparatus for performing a solid phase immunodiffusion assay in which a proteinaceous antibody or receptor is bound as a capture ligand to a membrane positioned over a hole at the bottom end. Bernstein operates by delivering ligand from the test sample to the capture membrane, whereby a ligand:receptor interaction is formed between the ligand and the capture agent and a signal is concentrated on the membrane sufficient to visualize the signal through a window 11. At least two objectives are fundamental to the teachings of Bernstein.

First, one of ordinary skill in the art in 1995, reading the Bernstein, Simpson, and Rich patents, would not have been motivated to provide a unit dose reagent chamber containing a luciferin-luciferase reagent by placing luciferin-luciferase in the vessel of the Bernstein apparatus, because the Bernstein apparatus is not suitable for chemiluminescent detection of ATP, and because modification of the Bernstein apparatus for chemiluminescent detection of ATP would have made the Bernstein apparatus unsuitable for its intended purpose of a solid phase immunodiffusion assay. Second Declaration of Dr. Steven J. Saul (Exhibit A, "Second Saul Declaration"), ¶9. The Bernstein apparatus is designed so that assay results can be observed visually through window 11, which is a discrete observation portal on the front side of lower portion 10. (See Figs. 5 of Bernstein). In fact, Bernstein states clearly that the Bernstein apparatus is designed to operate independently of instrumentation such as scintillation counters, flourometers and colorimeters. Bernstein, col. 1, para.

2; Second Saul Declaration, ¶12. To concentrate the signal in front of the window Bernstein must do four things: (a) capture the labeled members of the binding pair on capture membranes 18, 19; (b) eliminate interfering substances on a pre-filter membrane 25; (c) remove excess fluid on absorbent 17; and (d) deliver the reagents into direct proximity in front of the prefilter and reaction membranes. (See col. 5, lines 5-8: "The shape of the lower portion 10 is configured to enhance contact of the collection device tip with the pre-filter or reaction membranes.") Bernstein, col. 3, lines 34-51; Second Saul Declaration, ¶17. Absent each of these design features, Bernstein would not be able to achieve sufficient signal enhancement for visualization through the front window 11. Second Saul Declaration, ¶17.

It is an indicia of nonobviousness where, as here, the suggested combination of references would require a substantial reconstruction and redesign of the elements shown in the primary reference, as well as a change in the basic principle under which the primary reference construction was designed to operate. In order to modify the Bernstein device for use with luciferin-luciferase, the Bernstein device would have had to have been adapted for chemiluminescent detection, i.e., by modifying the devise to be suitable for use with a luminometer. Were the Bernstein device to have been so adapted, there would be no concentration of signal in front of window 11, and the Bernstein apparatus would then be unsatisfactory for its intended purpose. Second Saul Declaration, ¶¶18-20.

Second, one of ordinary skill in the art in 1995, reading the Bernstein, Simpson, and Rich patents, would not have been motivated to provide a unit dose reagent chamber containing a detergent-containing buffered solution for use in a test apparatus for detecting ATP in a test sample, by placing a detergent-containing buffered solution in the vessel of the Bernstein apparatus. The Bernstein apparatus features an open portal window and relies for its operation on the presence of a prefilter and capture membrane, and thus would not be suitable for use in ATP detection. Modification of the Bernstein apparatus to be suitable for ATP detection, by the substantial reconstruction of eliminating the prefilter and capture membrane, would result in leakage of unabsorbed fluid out of the window. Second Saul Declaration, ¶10 and ¶¶20-22.

Claims 7 and 10

For related reasons, the examiner has not established a *prima facie* case of obviousness with respect to claims 7 and 10. The inventions of claims 7 and 10 are directed to the combination of the unit dose reagent chamber and test apparatus of claim 5, along with a closed bottom end, transparent test unit at one end of the test apparatus. The bottom end of the Bernstein device, in contrast,

features window 11, and is not closed. Second Saul Declaration, ¶10 and ¶¶20-22; *see also,* Bernstein, claim 1, element h)(*"means forming a hole in said tube . . ."*). One skilled in the art would not be motivated to place a detergent containing buffered solution into the Bernstein apparatus. The bottom of the Bernstein device is not closed, so any solution would leak out the hole at the bottom of the test apparatus.

Claims 1, 2, and 12

The unit dose reagent chamber of claim 1 contains a detergent-containing buffered solution and/or a luciferin-luciferase reagent, for use in a test apparatus that would be suitable for performing an ATP detection assay that employs one or both of these reagents. To consider the invention as a whole, the rejection must account for the recitations of the preamble. *In re Mills*, 916 F.2d 680, 16 USPQ2d 1430, 1432 (Fed. Cir. 1990) (reversing Board's rejection of all claims as obvious where differences between reference and claims lie solely in the functional language in the preamble reciting an intended use for the machine).

When stating that,

> [t]he reference of Bernstein discloses a unit dose reagent chamber for use in a test apparatus (See Figure 4)"

the examiner ignores crucial claim limitations, and thus does not consider the claimed invention as a whole. *See,* Office Action, March 28, 2005, page 4, lines 708. The proper inquiry is not whether Bernstein discloses *"a unit dose reagent chamber for use in a test apparatus"*, but rather whether Bernstein discloses *"unit dose reagent chamber for use in a test apparatus for the detection of adenosine triphosphate (ATP) in a test sample"*. Bernstein does not disclose a test apparatus suitable for detecting detergent-released ATP by chemiluminescent reagents.

Secondary indicia of non-obviousness reduces the likelihood that one skilled in the art would be motivated to combine the prior art references to below the required 50% threshold..

A decision to maintain a rejection must show that the decision is based on the totality of the evidence. MPEP 2143.01. The examiner must consider not only those references cited, but also any and all evidence that supports patentability of applicant's invention, including any evidence of secondary considerations submitted by the applicant in rebuttal. *Id.* Evidence establishing a secondary indicia of non-obviousness can include evidence that others of ordinary skill in the relevant art arrived at alternative solutions. *Monarch Knitting Machinery Corp.*, 45 USPQ2d at

1983. General skepticism of those in the art that does not amount to "teaching away" is still relevant and persuasive evidence of non-obviousness. *Monarch Knitting Machinery Corp.*, 45 USPQ2d at 1984.

In the present case, there is at least a 50% likelihood that one skilled in the art, in possession of the cited references and seeking to solve the problem of detecting ATP using one or more of a detergent and a luciferin-luciferase reagent, would have chose *not* to modify the Bernstein test apparatus, but rather would have pursued an alternative solution. Evidence of the fact that one skilled in the art was at least as likely to pursue different options is found in US Patent 5,783,399, which was filed on November 17, 1995 by inventors Mary Ann Childs, Gregory K. Shipman, William P. Trainor, Erick Gray, and David Bernstein ("Childs et al.", Exhibit B).

We do not know what actually motivated Childs et al. but even if we did it would be irrelevant to the issue of obviousness. *Amazon.com, Inc. v. Barnesandnoble.com, Inc.*, 239 F.3d 1343, 57 U.S.P.Q.2d 1747 (Fed.Cir. 2001). The relevant inquiry is what a hypothetical ordinarily skilled artisan would have gleaned from the cited references at the time of the invention. *Id.* The written disclosure of the Childs et al. patent is *prima facie* evidence establishing that it is just as likely that the skilled artisan, seeking to solve the problem of providing a device for chemiluminescent detection of detergent-released ATP in a test sample, would have arrived at a completely different solution than that encompassed by the claimed invention. Given evidence of an alternative solution by those skilled in the art in 1995, a *prima facie* case of obviousness has not been established to a preponderance of the evidence. *Monarch Knitting Machinery Corp.*, 45 USPQ2d at 1982 (reversing the district court's finding of obviousness where *"All of these references stated the problem as preventing hook breakage at high speeds. Each of these references proposed a different solution. Thus, this evidence creates a genuine issue as to whether those of ordinary skill would have had a motivation to combine needles with varying stem segment heights to form a trend."*).

The inventors of the Childs et al. patent sought to use chemiluminescent methods to detect ATP when monitoring surfaces for bacterial contamination. Exhibit B, col. 1, lines 6-56. Childs et al. recognized that luciferin-luciferase reactions of the firefly had been used previously for detecting threshold levels of microorganisms. Exhibit B, paragraph bridging col. 1-2. But Childs et al. also recognized that lyophilized luciferase-luciferin reagent could be unstable at room temperature during

long term storage, and considered it to be unstable after liquid reconstitution over short time intervals. *Id.*

Given that all of the cited references of Bernstein, Simpson, and Rich predated the filing date of Childs et al., applicant is entitled to the legal presumption that the cited references were available to Childs et al. , especially in view of their collaborative relationship with co-inventor David Bernstein. Yet when faced with the problem of using luciferin-luciferase to detect ATP on a test surface as an indication of bacterial contamination, the inventors of the Childs patent did not choose to modify the Bernstein reference. The inventors on the Childs patent chose a completely different solution to that problem.

The device that Childs et al. discloses for accomplishing this goal is a lateral flow type device having a lateral flow membrane on a solid support strip, having a sample portion, a reagent portion, and a fluid reservoir on the test strip that breaks in response to finger pressure to cause carrier fluid to flow from the reservoir across the lateral flow strip. The solution arrived at by Childs et al. included either drying a detergent onto the sample portion or reagent portion of the test strip (col. 5, lines 7-10 and 61-65), or placing a detergent into the carrier fluid in the fluid reservoir (col. 3, lines 31-33 and col. 6, lines 4-5). The solution of Childs et al. further includes applying a reconstituted solution of luciferin-luciferase to the membrane filter strip and drying *in vacuo* (col. 8, lines 49-56).

Thus, the Childs et al. patent makes it clear that, despite possession of the cited references, one of skill in the art would not necessarily have arrived at the solution of providing a unit dose reagent chamber containing either a detergent-containing buffered solution or a luciferin-luciferase reagent. It has not been established to a preponderance of the evidence, i.e., that it is more than 50% likely, that one skilled in the art would have been motivated to modify or combine Bernstein, Simpson, and Rich to arrive at the unit dose reagent chamber, test apparatus, and test unit of applicant's claimed invention.

In light of the above, applicant submits that the rejection can not be maintained, because the rejection lacks rationale as to why the skilled artisan would modify the Bernstein apparatus in keeping with the disclosures of Simpson and Rich, given that such modifications would have required substantial reconstruction of the Bernstein apparatus, changed its principle of operation, and rendered it unsuitable for its intended purpose. In addition, the Childs et al. patent is evidence that those skilled in the art, in possession of the same prior art references in 1995 and seeking to solve the

problem of providing a device for chemiluminescent detection of ATP in a test sample, would have been just as likely to seek a completely different solution. Thus, the totality of the evidence does not support a conclusion that there is a greater than 50% likelihood that the claimed invention would have been obvious to one skilled in the art in view of Bernstein, Simpson, and Rich.

II. Rejection under 35 U.S.C. § 103(a): Bernstein in view of Simpson, Rich, and Matsumoto

Claims 10, 14, 15, 17-19, 23, 24 and 26 have been rejected under 35 U.S.C. § 103(a) as not being patentable over Bernstein (US 4,770,853) in view of Simpson et al. (EP 0 309 184; "Simpson") and Rich et al. (US 3,666,631; "Rich") and taken further in view of Matsumoto et al. (JP 7-59555). Applicant respectfully requests that the rejection be reversed.

Matsumoto et al. is not directed to a separate sealed reagent chamber or to the use of multiple, aligned reagent chambers in a test unit. The Matsumoto et al container is a pocket-type, microbial incubator, not a reagent test apparatus. The Matsumoto et al. container provides for the separation of a "liquid substance containing an indicator" in a sealed package, from a dry, inactive microorganism tablet in the bottom end of the incubator. The Matsumoto et al. incubator solves the problem of keeping the dry microorganism apart from the liquid until the collecting bead with the sample is used to inoculate the moistened microorganisms.

The Matsumoto et al. apparatus is not directed to the detection of ATP or AP in a luminescent method within the test unit and does not have or suggest single or multiple, separate, aligned unit dose reagent chambers in a test unit, nor does it have a transparent test unit, with a reagent chamber to be removedly secured from one end of the test unit for separate insertion into a photometer for observation (see application Figs. 5 and 7).

The Bernstein, Simpson, and Rich references have been discussed above. The above arguments and the testimony set forth in the Second Saul Declaration relating to the Bernstein, Simpson, and Rich references apply equally to the invention of claims 10, 14, 15, 17-19, 23, 24, and 26, and are hereby incorporated by reference. In addition, Childs et al., US Patent 5,783,399 (Exhibit B) is secondary indicia that one skilled in the art, having possession of Bernstein, Simpson, Rich, and Matsumoto, would not have been motivated to arrive at the solution of the claimed invention.

All of the cited references of Bernstein, Simpson, Rich, and Matsumoto predated the filing date of Childs et al. Applicant is thus entitled to the legal presumption that the cited references were

available to Childs et al., especially in view of their collaborative relationship with co-inventor David Bernstein. Although Childs et al. sought to provide a device for detecting ATP on a test surface as an indication of bacterial contamination, the inventors of the Childs patent did not choose to modify the apparatus of the primary Bernstein reference according to the disclosures of Simpson, Rich, and Matsumoto. The inventors on the Childs patent chose a completely different solution to that problem, namely, a lateral flow diffusion assay.

Childs et al. establishes that there is at least a 50% likelihood that one skilled in the art, in possession of the cited references and seeking to solve the problem of detecting ATP using one or more of a detergent and a luciferin luciferase reagent, would have chosen ***not*** to modify the Bernstein test apparatus, but rather to have pursued an alternative solution. Thus, the examiner has not established that those skilled in the art, in possession of the cited references, would have necessarily arrived at the claimed invention. Other options were available, creating an at least 50% probability that that one skilled in the art could have would have come up with an alternative solution to that of claims 10, 14, 15, 17-19, 23, 24, and 26.

Respectfully submitted:

Date: Oct 12, 2006

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VIII. Claims Appendix

1. (twice amended) A unit dose reagent chamber for use in a test apparatus for the detection of adenosine triphosphate (ATP) [or alkaline phosphatase (AP)] in a test sample, and wherein a moveable probe is employed to obtain a test sample and to release reagents from the reagent chamber to a test unit, which unit dose chamber comprises:

a) a cylinder having a one open end and an other opposite open end;

b) a probe-puncturable membrane seal over the one end and the other end of the cylinder to form a sealed compartment; and

c) a reagent composition [for use in the detection of the test sample and sealed] within the sealed compartment, which composition consists essentially of and is selected from the group consisting of:

i) a detergent-containing buffered solution to release adenosine triphosphate (ATP) [or alkaline phosphatase (AP)] from the test sample into the solution for testing; and

ii) [a reaction stopping solution having a pH of 8 to 11; and

iii)] a luciferin-luciferase [or phosphatase substrate] reagent [tablet].

2. (original) The chamber of claim 1 wherein the membrane seal comprises aluminum foil.

3. (cancelled).

4. (twice amended) A unit dose reagent [The] chamber [of claim 1] for use in a test apparatus for the detection of adenosine triphosphate (ATP) in a test sample and wherein a moveable probe is employed to obtain a test sample and to release reagents from the reagent chamber to a test unit, which unit dose chamber comprises;

a) a cylinder having a one open end and an other opposite open end;

b) a probe-puncturable membrane seal over the one end and the other end of the cylinder to form a sealed compartment; and

c) a reagent composition within the sealed compartment, which composition consists essentially of and is selected form the group consisting of;

wherein the reagent composition is selected from the group consisting of (i) a detergent-containing buffered solution to release adenosine triphosphate (ATP) from the test sample into the solution for testing; and (ii) a luciferin-luciferase reagent, and wherein the reagent composition includes a pH indicator.

5. (twice amended) In combination, the chamber of claim 1 in a test apparatus for the detection of adenosine triphosphate (ATP) [or alkaline phosphatase (AP)] in a test sample, wherein the reagent composition is a detergent-containing buffered solution to release adenosine triphosphate (ATP) from the test sample into the solution for testing, which test apparatus includes a luciferin-luciferase [or phosphatase substrate] reagent for reaction with the released adenosine triphosphate (ATP) [or alkaline phosphatase (AP)] in the solution.

6. (amended 2/03) The combination of claim 5 wherein the test apparatus [includes] further comprises a longitudinally moveable probe to puncture the membrane seals[to carry out the test].

7. (amended) The combination of claim 5 wherein the test apparatus [includes] further comprises a closed bottom end, transparent test unit at the one end of the test apparatus, and wherein one or more unit dose reagent chambers are longitudinally positioned in the test unit.

8. (cancelled).

9. (cancelled).

10. (three times amended) The combination of claim 7 wherein the reagent composition is a detergent-containing buffered solution to release adenosine triphosphate (ATP) from the test sample into the solution for testing [sealed compartment comprises the buffered-detergent solution] and wherein said test apparatus includes a luciferase and a luciferin reagent [in tablet form] at the bottom end of the test unit.

11. (cancelled).

12. (twice amended) The chamber of claim 1, wherein the reagent composition is selected from the group consisting of i) a detergent-containing buffered solution to release adenosine triphosphate (ATP) from the test sample into the solution for testing; and ii) a luciferin-luciferase reagent, and wherein the reagent composition includes a biological buffer solution to optimize a reaction for the detection of adenosine triphosphate (ATP) [or alkaline phosphatase (AP)].

13. (cancelled).

14. (three times amended) A test apparatus for the detection of adenosine triphosphate (ATP) [or alkaline phosphatase (AP)] in a test sample, by luminescence [or color], which test apparatus comprises:

a) a longitudinal test apparatus housing having a one end and an other end;

b) a moveable probe within the housing to collect a test sample and arranged to puncture a membrane seal;

c) a transparent test unit having a one end and a closed bottom end extending from the one end of the housing for use in detecting luminescence [or color] in the test sample, and a first reagent

[tablet] composition to detect adenosine triphosphate (ATP) [or alkaline phosphatase (AP)], by [color or] luminescence, at the closed bottom end; and

d) one or more unit dose reagent chambers longitudinally-positioned in the test unit, which reagent chamber comprises:

i) a cylinder having a one open end and an other opposite open end;

ii) a probe-puncturable membrane seal at and over the one end and the other end of the cylinder to form a sealed compartment; and

iii) a second reagent composition for use in the detection of adenosine triphosphate (ATP) [or alkaline phosphatase (AP)] in the test sample and sealed within the sealed compartment, which reagent composition comprises a buffered solution to release adenosine triphosphate (ATP) [or alkaline phosphatase (AP)] from the test sample into the solution for subsequent reaction with the first reagent [tablet] composition.

15. (original) The apparatus of claim 14 wherein the membrane seal comprises aluminum foil.

16. (cancelled).

17. (twice amended) The apparatus of claim 14 wherein the test unit has an open top end [with threads] and a closed bottom end and is detachably [removedly, threadably] secured to one end of the test apparatus.

18. (original) The apparatus of claim 14 wherein the one end of the test unit is sealed with a probe-puncturable membrane.

19. (twice amended) The apparatus of claim 14 wherein the sealed compartment comprises a buffer-detergent solution and a luciferase and a luciferin substrate, as a reagent [tablet], is at the bottom end of the test unit.

20. (cancelled).

21. (cancelled).

22. (cancelled).

23. (twice amended) A transparent test unit for use in a test apparatus for the detection of a test sample, which test unit comprises: a one end; a closed bottom end; a probe-puncturable membrane over the one end; and the one end having means for detachably securing the test unit to the test apparatus and the test unit having one or more unit dose reagent chambers, which unit dose chamber comprises:

a) a cylinder having a one open end and an other opposite open end;

b) a probe-puncturable membrane seal over the one end and the other end of the cylinder to form a sealed compartment: and

c) a reagent composition for use in the detection of the test sample and sealed within the sealed compartment; and [The test unit of claim 21] wherein the test unit includes a luciferin-luciferase reagent [tablet].

24. (added; amended) The apparatus of claim 19, wherein said luciferase and said luciferin reagent are in tablet form.

25. (cancelled).

26. (added) The test unit of claim 23, wherein said luciferin-luciferase reagent is a luciferin-luciferase tablet.

27. (added; cancelled).

28. (added; cancelled).

29. (added; cancelled).

30. (added; amended) A unit dose reagent chamber for use in a test apparatus for the detection of alkaline phosphatase (AP) in a test sample, and wherein a moveable probe is employed to obtain a test sample and to release reagents from the reagent chamber to a test unit, which unit dose chamber comprises:

a) a cylinder having a one open end and an other opposite open end;

b) a probe-puncturable membrane seal over the one end and the other end of the cylinder to form a sealed compartment; and

c) a reagent composition within the sealed compartment, which composition consists essentially of and is selected from the group consisting of: i) a detergent-containing buffered solution to release alkaline phosphatase (AP) from the test sample into the solution for testing; and ii) a reaction stopping solution having a pH of 8 to 11; and wherein the reagent composition includes a pH indicator.

Claims 31-45 (added; cancelled).

IX. Evidence Appendix

The following evidence was submitted in the application pursuant to U.S.C §§ 1.130, 1.131, or 1.132, and is relied on in the present appeal.

Exhibit No.	Evidence	Statement setting forth where in the record evidence was entered in the record by the Examiner
A	Second Declaration of Dr. Steven J. Saul ("Second Saul Declaration")	Entered with Applicant's Amendment filed 9/28/05, as Exhibit A thereto
B	Childs et al., US Patent 5,783,399, issued July 21, 1998	Entered with Applicant's Amendment filed 9/28/05, as Exhibit B thereto
C	Declaration of Dr. Steven J. Saul under 37 C.F.R. § 1.132 ("First Saul Declaration")	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B thereto
C1	Curriculum vitae of Dr. Steven J. Saul (Exhibit 1 to First Saul Declaration)	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B1 thereto
C2	Stanley, P.E., *Extraction of Adenosine Triphosphate from Microbial and Somatic Cells, Methods in Enzymology*, 133:14-22 (1986) (Exhibit 2 to First Saul Declaration)	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B2 thereto
C3	Andreotti et al., WO 92/20781, November 26, 1992 (Exhibit 3 to First Saul Declaration)	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B3 thereto
C4	Wood, US Patent No. 5,283,179, 1994 (Exhibit 4 to First Saul Declaration)	Entered with Amendment filed 3/25/04, as Exhibit B4 thereto
C5	Optimization of the Firefly Luciferase Assay for ATP (Webster, J.J., and Leach, F.R., *Jour. Applied Biochemistry*, 2:469-479, 1980) (Exhibit 5 to First Saul Declaration)	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B5 thereto
C6	Effect of Solvents on the Catalytic Activity of Firefly Luciferase (Kricka, L.J. et al., *Archives of Biochemistry and Biophysics*, 217(2), 1982) (Exhibit 6 to First Saul Declaration)	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B6 thereto
C7	U.S. Patent No. 5,004,684, Simpson et al, 1991 (Exhibit 7 to First Saul Declaration)	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B7 thereto

Exhibit No.	Evidence	Statement setting forth where in the record evidence was entered in the record by the Examiner
C8	US Patent No. 4,303,752, Kolehmainen et al., 1981 (Exhibit 8 to First Saul Declaration)	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B8 thereto
C9	Denville Scientific Inc., *Research Products Catalog 1991,* pages 1, 2, 15 (Exhibit 9 to First Saul Declaration)	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B9 thereto
C10	Denville Scientific Inc., *Research Products Catalog,* 1995, pages 1, 2, 16 (Exhibit 10 to First Saul Declaration)	Entered with Applicant's Amendment filed 3/25/04, as Exhibit B10 thereto
D	U.S. Provisional Application 60/001,081, filed July 12, 1995	
E	U.S. Provisional Application 60/007,585, filed November 27, 1995	
F	Examiner's Interview Summary, March 23, 2006.	Examiner's Interview Summary, March 23, 2006.

X. Related Proceedings Appendix

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Skiffington et al.	Examiner:	Beisner, William H.
Reissue Serial No:	10/014,154	Art Unit:	1744
Filed:	December 6, 2001		
Original Patent:	6,180,395		
Original Patent Issue Date:	January 30, 2001		
Title:	Reagent Chamber For Test Apparatus and Test Apparatus		

SECOND DECLARATION OF DR. STEVEN J. SAUL

I declare:

1. I have been employed in the area of scientific research and development by Charm Sciences, Inc., the assignee of the above-captioned patent application, since 1990. I currently hold the position of Director of Research.

2. I have worked and published extensively in the area of protein chemistry and enzymatic reactions, and I am an expert in this field. In 1983, I was awarded a PhD degree in Biological Science, with an emphasis in enzymology, from the University of Rhode Island, RI. From 1984-1990, I conducted postdoctoral studies in several areas of enzymatic biochemistry, which included the protein biochemistry and reaction mechanisms of several insect systems. Attached, as Exhibit 1 hereto is my curriculum vitae, which lists over 32 publications in this scientific area of which I am an author or a co-author. My curriculum vitae further include seven issued patents in this scientific area of which I am an inventor or co-inventor. Exhibit 1.

3. I have extensive experience using and refining techniques for detecting ATP by the luciferin-luciferase enzymatic reaction, and with the use of lysis solutions to extract

ATP from cellular samples. I hold three U.S. patents which concern subject matter relating to the use of the luciferase enzyme with various derivatives of luciferin. See, Exhibit 1, page 3.

4. I have read the patent application US Serial No. 10/014,154, and have also read the Office Action issued on March 28, 2005.

5. I have reviewed the disclosure of US Patent 4,770,853, which issued on September 13, 1988, to David Bernstein of Sykesville, Maryland.

6. I have reviewed the disclosure EP 0309184, Simpson et al, 1991, "Method for ATP Extraction" (hereafter "Simpson").

7. I have reviewed the disclosure of US Patent 3,666,631, which issued on May 30, 1972, to Rich et al (hereafter "Rich").

8. I have reviewed the disclosure of Matsumoto et al., JP 7-59555 (hereafter "Matsumoto").

9. It is my opinion that one of ordinary skill in the art in 1995, reading the Bernstein, Simpson, and Rich patents, would not have been motivated to provide a unit dose reagent chamber containing a luciferin-luciferase reagent by placing luciferin-luciferase in the vessel of the Bernstein apparatus, because the Bernstein apparatus is not suitable for chemiluminescent detection of ATP, and because modification of the Bernstein apparatus for chemiluminescent detection of ATP would have made the Bernstein apparatus unsuitable for its intended purpose of a solid phase immunodiffusion assay.

10. It is my opinion that one of ordinary skill in the art in 1995, reading the Bernstein, Simpson, and Rich patents, would not have been motivated to provide a unit dose reagent chamber containing a detergent-containing buffered solution for use in a test

apparatus for detecting ATP in a test sample, by placing a detergent-containing buffered solution in the vessel of the Bernstein apparatus. The Bernstein apparatus features an open portal window and relies for its operation on the presence of a prefilter and capture membrane, and thus would not be suitable for use in ATP detection. Modification of the Bernstein apparatus to be suitable for ATP detection, by the substantial reconstruction of eliminating the prefilter and capture membrane, would result in leakage of unabsorbed fluid out of the window.

11. Based on my review of the Bernstein patent, it is my view that Bernstein sought to provide a test device suitable for performing a ligand receptor assay to detect antigens, haptens, antibodies, DNA or RNA fragment, wherein the user is not required to dispense any of the reagents. Further design criteria were that all reagents be self-contained within a device that could be stored at nonrefrigerated temperatures, and which could utilize lyophilized reagents. Bernstein, column 2, line 56, to column 3, line 2.

12. I further note that, at column 2, lines 46-55, Bernstein states that it is an object to transfer the reactants "to a reaction zone where the specific labeled reactant can be captured and visualized." I further note that, at column 1, paragragh 2, the Bernstein patent expresses the goal of eliminating any need for capital equipment such as "scintillation counters, flourometers and colorimeters in the case of radioimmunoassay, fluorescent immunoassay, and enzyme immunoassay respectively".

13. It is my opinion that the Bernstein apparatus is constructed so as to accomplish the goal of performing a rapid solid phase immunodiffusion assay. At column 3, lines 49-52,

Bernstein states that "[t]he configuration of the lower portion allows the collection device to come into physical contact with the prefilter, capture membrane or capture filter."

14. At column 2, lines 26-29, Bernstein states that "[i]n the case where membranes or filters are used to capture the immunoreactants, it is necessary to bring the fluid containing the immunoreactants in contact with the filter or membrane."

15. At column 2, lines 46-55, Bernstein further articulates the importance of having a larger pore size filter or membrane between the swab and capture membrane to retain any unwanted cells or debris that may interfere with the assay.

16. The Bernstein apparatus is also configured so that the assay results can be observed visually through a window, which is a discrete observation portal on the front side of the lower portion of the device. In order to visualize the signal without the aid of capital equipment, it was necessary to concentrate the signal in front of the window.

17. To concentrate the signal in front of the window Bernstein had to do four things: (a) capture the labeled members of the binding pair on capture membranes 18, 19; (b) eliminate interfering substances on a pre-filter membrane 25; (c) remove excess fluid on absorbent 17; and (d) deliver the reagents into direct proximity in front of the prefilter and reaction membranes. Bernstein, column 3, lines 34-51. I also note that, at column 5, lines 5-8, Bernstein states, "The shape of the lower portion 10 is configured to enhance contact of the collection device tip with the pre-filter or reaction membranes." Absent each of these design features, Bernstein would not be able to achieve sufficient signal enhancement for visualization through the front window 11.

18. It is my opinion that it would not have been obvious to one of ordinary skill in the art in 1995 to modify the Bernstein apparatus to be suitable for chemiluminescent detection of ATP with luciferin-luciferase. Adaptation of the Bernstein apparatus for chemiluminescent detection would have required modification of the device to be suitable for use with a luminometer. Those skilled in the art would not have found a suggestion or motivation to modify the Bernstein apparatus for use with a luminometer. To do so would have contradicted Bernstein's goal of providing a rapid immunodiagnostic assay that operated independently of capital equipment.

19. Another reason those skilled in the art would not have adopted the Bernstein apparatus for use with a luminometer is that, were one to do so, the Bernstein apparatus would have become inoperable for its intended purpose of visualization of signal by the naked eye. The shape of the lower portion of the Bernstein apparatus is configured to enhance contact of the collection device tip with the prefilter or reaction membranes. Bernstein, column 5, lines 5-7. Were the lower portion of the Bernstein apparatus to be modified to fit inside a luminometer, its shape would no-longer be configured to enhance contact of the collection tip with the prefilter or reaction membranes.

20. One skilled in the art would not have been motivated to modify the Bernstein apparatus to operate without prefilter or reaction membranes. Were the Bernstein apparatus to have been so adapted, there would be no concentration of signal in front of window 11, and the Bernstein apparatus would then be unsatisfactory for its intended purpose.

21. It is my opinion that one skilled in the art in 1995 would not have been motivated to place a detergent-containing buffered solution into the vessel of the Bernstein

device, because the Bernstein apparatus does not contain a closed bottom end. I note that, at column 5, lines 15-25, Bernstein describes an "adhesive tape 12 that holds the absorbent [17] in place and applies the necessary pressure to ensure diffusion of fluid through the various layers of the ligand receptor test area." The absorbent 17 absorbs excess fluid diffusing through the membranes. By removing adhesive tape 12 by lifting tab 28 of Bernstein, the bottom end of the Bernstein device is not a closed bottom end.

22. It is my opinion that one skilled in the art would not be motivated to undergo the substantial reconstruction of the Bernstein device that would be required to make it suitable for detecting ATP using a detergent-containing buffered solution. The bottom of the Bernstein device is not closed, so any solution would leak out the "window."

23. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Steven J. Saul, Ph.D

Date: 9/28/05

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1984 - 1985	Post-doctoral fellow, Children's Hospital, Boston
1979 - 1982	Instructor, University of Rhode Island
1977 - 1980	Graduate Res. Asst., University of Rhode Island

Publications:

1. Saul SJ: The Metabolism of Sesamol by Rat Liver Microsomes and the Role of Cytochrome b5 and the Mixed Function Oxidase System in Sesamol Metabolism. Ph.D. Dissertation, University of Rhode Island, (1983).
2. Sugumaran M, Saul SJ, and Ramesh N: Endogenous Protease Inhibitors Prevent Undesired Activation of Prophenolase in Insect Hemolymph. Biochem. Biophys. Res. Commun. 2 , 1124-1129 (1985).
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26. Saul, S. Boyer, C. Markovsky, B. Salter, R. Lawton-Scheemaker, J. and Charm S. The New Charm SL (Safe Level) ß-lactam Test Significantly Reduces Rejection of Milk Positive by Other Screening Methods. National Mastitis Council ; 1999 Annual Meeting Proceedings.

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1. Saul SJ and Sugumaran M: Phenoloxidase activation, proteases and protease inhibitors in insect Hemolymph. Fed. Proc. Fed. Amer. Soc. Exptl. Biol. 44, 1860 (1986)

2. Saul SJ and Sugumaran M: Activation of two different phenoloxidases in the hemolymph of Sarcophaga bullata larvae in response to zymosan treatment.Proc. XVIII Int. Congr. Entomol. p. 143 (1988)

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4. Saul, SJ. Zomer, E and Charm SE: Charm Pesticide Test: Rapid Screening Method for the Detection of Organophosphate and Carbamate Pesticides for Water, Dairy Products, Fruits,Vegetables and Other Food Products. IAMFES. 80th Annual Meeting . 17-18, (1993)

Patents:

1. Zomer, E. Saul, S. and Charm, SE: Bioluminescence Method for the Determination of Pesticides.US Patent Number: 5,283,180. (1994)

2. Zomer, E. Saul, S. and Charm, SE: Method of Preparing D-Luciferin Derivatives, US Patent Number 5,374,534. (1994)

3. Zomer, E. Saul, S. and Charm, SE: Test kit for Determination of Organophosphate and Carbamate with Insect Brain Material That Hydrolyses a 6-Substitutes D-Luciferin Ester. US Patent Number 5,374,535. (1994)

4. Charm, SE. Skiffington, R. Markovsky, RJ. Zomer, E. and Saul SJ: Test Device for Detection of an Analyte. US patent Number 5,985,675. (1999) (note: see certificate of correction)

5. Markovsky, RJ. Boyer, CA. Charm SE. Donahue, PR. Glickman, YA, Saul, SJ. Scheemaker, JL. Skiffington, RT. Trivedi, ST and Zomer, E. Test Device for Detecting the Presence of a Residue Analyte in a Sample. US Patent Number 6,319,466. (2001)

6. Charm SE. Skiffington, R. Markovsky RJ. Zomer E. and Saul, SJ: Method for Detection of an Analyte. US Patent Number 6,475,805. (2002)

7. Zomer, E. Saul, S and Charm, SE; Test Kit and Method for the Determination of Pesticides. EP Patent Number 0576667. (2001)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Skiffington et al.	Examiner:	Beisner, William H.
Reissue Serial No:	10/014,154	Art Unit:	1744
Filed:	December 6, 2001		
Original Patent:	6,180,395		
Original Patent Issue Date:	January 30, 2001		
Title:	Reagent Chamber For Test Apparatus and Test Apparatus		

DECLARATION OF DR. STEVEN J. SAUL UNDER 37 C.F.R. §1.132

I declare:

1. I have been employed in the area of scientific research and development by Charm Sciences, Inc., the assignee of the above-captioned patent application, since 1990. I currently hold the position of Director of Research.

2. I have worked and published extensively in the area of protein chemistry and enzymatic reactions, and I am an expert in this field. In 1983, I was awarded a PhD degree in Biological Science, with an emphasis in enzymology, from the University of Rhode Island, RI. From 1984-1990, I conducted postdoctoral studies in several areas of enzymatic biochemistry, which included the protein biochemistry and reaction mechanisms of several insect systems. Attached, as Exhibit 1 hereto is my curriculum vitae, which lists over 32 publications in this scientific area of which I am an author or a co-author. My curriculum vitae further include seven issued patents in this scientific area of which I am an inventor or co-inventor. Exhibit 1.

3. I have read the Office Action issued in U.S. Serial No. 10/014,154 on September 25, 2004, and I have read provisional patent application 60/001,081, filed July 12, 1995 (hereafter "the '081 provisional").

4. I have extensive experience using and refining techniques for detecting ATP by the luciferin-luciferase enzymatic reaction, and with the use of lysis solutions to extract ATP from cellular samples. I hold three U.S. patents which concern subject matter relating to the use of the luciferase enzyme with various derivatives of luciferin. See, Exhibit 1, page 3.

5. I note that the '081 provisional, e.g., in the second paragraph and in figure 2 describes *inter alia*, "microbialysis [sic] solution and an ATP stabilizer, a buffer optimized for luciferin-luciferase reaction, . . . " "microbial lysis solution," and "buffer optimized for luciferin-luciferase reaction." The meanings of terms such as "microbial lysis solution", "ATP stabilizer", and "buffer optimized for luciferin-luciferase reaction" were conventional and commonly understood by those of ordinary skill in the art in 1995, with such meanings reflected in the literature of the time.

6. By July of 1995, methods and reagents for extracting ATP from cellular samples with lysis solutions were well known and commonplace, and the luciferin-luciferase assay was a well-established and conventional technique for detecting such ATP. In fact, by 1995 it was commonly understood that a main reason for extracting ATP was as a basis for subsequently measuring that ATP by the luciferin-luciferase assay. See, page 14-15 of Stanley, P.E., *Extraction of Adenosine Triphosphate from Microbial and Somatic Cells, Methods in Enzymology*, 133:14-22, Academic Press, London 1986) (hereafter "the Stanley review". The Stanley review is attached as Exhibit 2 hereto.

7. Referring to the first paragraph of the '081 provisional, one skilled in the art in July 1995 would have understood the passage "a portable pocket-swab type test kit and method for detecting the presence of ATP on surfaces in water and other biological fluids or foods . . " to refer to a device for detecting a mixed population of cellular contaminants on surfaces and in samples of water, biological fluids, and foods. Stanley, a 1986 review article, points out that cells are commonly present as a mixed population in nonliving

materials such as soil, meat, milk, fruit juices, and clinical samples. Exhibit 2, at page 21.

8. It is my opinion that one skilled in the art in July 1995 would have understood the '081 provisional to have described a reagent composition that included a buffered solution to release adenosine triphosphate (ATP) from the test sample into the solution for subsequent testing by the luciferin-luciferase assay. In fact, it has long been well known and established that ATP release reagents were known to include a buffer composition. For example, the conventional practice of using buffered lysis solutions to extract ATP from cellular samples is reflected in the background section of a published PCT patent application, Andreotti et al., WO 92/20781, November 26, 1992 (hereafter "Andreotti," Exhibit 3 hereto). Andreotti summarizes various then-known ATP releasing reagents, pointing out that ATP releasing reagents were commonly combined with buffers to adjust pH. Exhibit 3, pages 1-3. It was thus well known and established, prior to Andreotti, that ATP release reagents were known to include a buffer composition. Exhibit 3, page 2-3.

9. US Patent No. 5,283,179, Wood, 1994, "Luciferase Assay Method" (hereafter "Wood", Exhibit 4 hereto) provides another example of a buffered lysing solution optimized for extracting ATP and detecting ATP with luciferase enzyme. One solution in Wood consisted of a Tris-phosphate buffer, glycerol or ethylene glycol, Triton X-100, bovine serum albumin (BSA), cyclohexylenediaminetetraacetate (CDTA), and DTT. Exhibit 4, col. 16, lines 10-18. More generally, Wood teaches that typical buffering agents were known to include, e.g., tricine, HEPPS, HEPES, MOPS, Tris, glycylglycine, and a phosphate salt to maintain pH and ionic strength. Exhibit 4, col. 8, lines 14-25.

10. Methods for optimizing buffered solution conditions in order to detect ATP by the luciferin-luciferase assay were well established, conventional, and routine in 1995. Guidance for optimizing conditions for the luciferin-luciferase assay were widely available in the form of review articles in journals that were widely read by those skilled

in the art. One example is Optimization of the Firefly Luciferase Assay for ATP (Webster, J.J., and Leach, F.R., *Jour. Applied Biochemistry*, 2:469-479, Academic Press, London, 1980) (hereafter "Webster", attached as Exhibit 5 hereto). Webster summarizes the relative affect of various buffers on luciferase activity. Exhibit 5., pages 469-470 and Table I.

11. Stanley is a second example of such a review article. Exhibit 2. The Stanley review is a summary of what was already known in the field pertaining to the properties of the ideal extractant, including those properties that were known to have an affect on the ATP-firefly luciferase assay. Exhibit 2, pages 15 and 16. Stanley cites as examples two detergents that were commonly used in 1986: Triton-X-100 and benzalkonium chloride. Considering the four categories of main extractants described by Stanley (Exhibit 2, page 18), one skilled in the art in 1995 would invariably have concluded that a detergent would be the most appropriate for the ATP-firefly luciferase application.

12. A third review article stressing the importance of detergents to the ATP-luciferase reaction is the 1982 review article Effect of Solvents on the Catalytic Activity of Firefly Luciferase (Kricka, L.J. et al., *Archives of Biochemistry and Biophysics*, 217(2), Academic Press, London 1982) (hereafter "Kricka," Exhibit 6 hereto). Kricka describes how a variety of detergents stimulate and/or inhibit the ATP-luciferase reaction. Exhibit 6, page 676.

13. One skilled in the art in July 1995 would have known a detergent to be a necessary component of a lysis solution for releasing ATP into solution in preparation for testing with luciferin-luciferase. For example, U.S. Patent No. 5,004,684, Simpson et al, 1991, "Method for ATP Extraction" (hereafter "Simpson", Exhibit 7 hereto) demonstrates the understanding of those skilled in the art of the importance of using detergent as an extractant. In particular, Simpson uses a detergent-containing buffered solution to release ATP into the test solution for testing, making multiple references to extracting ATP from microorganisms using detergents in buffers that had been optimized

for the luciferin-luciferase assay. Simpson, col. 3-4. In fact, Simpson refers to the Stanley review when citing certain disadvantages to using acids and organic solvents (Simpson, col. 2, lines 49-55). Thus, by 1995 one skilled in the art would have understood that ample guidance was available for determining which detergents and in what concentrations.

14. Further illustration of the use of detergents as lysing agents when the '081 provisional was filed is shown by the summary of the state of the art by Andreotti, and by the patents of Wood and Kolehmainen et al. (Exhibits 3, 4, and 8). Wood referred to the use of detergents or surfactants as being routine. Exhibit 4, col. 8, lines 36-43. Andreotti summarized the work of a conventionally known and oft cited patent in the field of ATP extraction and detection, US Patent No. 4,303,752, Kolehmainen et al., 1981 (Exhibit 8, attached hereto). The Kolehmainen patent refers to both ionic surfactants and non-ionic surfactants for use in releasing ATP from cells. Exhibit 8.

15. The term 'microtube' was commonly used by 1995 to refer to a microcentrifuge tube. Exhibits 9 and 10. The depiction of the microtube test unit shown in the drawings of the '081 provisional would have conveyed to one skilled in the art in 1995 the option of using threads as a means of attaching the microtube to a test apparatus. Attached hereto are pages from two standard commercial catalogs: Denville Scientific Inc., *Research Products Catalog 1991,* pages 1, 2, 15 (Exhibit 9) and Denville Scientific Inc., *Research Products Catalog,* 1995, pages 1, 2, 16, (Exhibit 10), both of which were readily available and commonplace before and during 1995. It is clear from page 15 of Exhibit 9 and from page 16 of Exhibit 10 that one skilled in the art would have understood a microtube having the appearance of the microtube in the drawings of the '081 provisional to match the microtubes shown in these catalogs, which are screw cap microtubes. Exhibits 9 and 10.

16. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and

further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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1984 - 1985	Post-doctoral fellow, Children's Hospital, Boston
1979 - 1982	Instructor, University of Rhode Island
1977 - 1980	Graduate Res. Asst., University of Rhode Island

Publications:

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2. Sugumaran M, **Saul SJ**, and Ramesh N: Endogenous Protease Inhibitors Prevent Undesired Activation of Prophenolase in Insect Hemolymph. Biochem. Biophys. Res. Commun. 2 , 1124-1129 (1985).
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26. Saul, S. Boyer, C. Markovsky, B. Salter, R. Lawton-Scheemaker, J. and Charm S. The New Charm SL (Safe Level) ß-lactam Test Significantly Reduces Rejection of Milk Positive by Other Screening Methods. National Mastitis Council ; 1999 Annual Meeting Proceedings.

27. Salter, R. Legg, D. Ossana, N. Boyer, C. Scheemaker, J. Markovsky, R. and Saul, SJ: Charm Safe-Level ß-Lactam Test for Amoxicillin, Ampicillin, Ceftiofur, Cephapirin, and Penicllin G in Raw Commingled Milk. J. AOAC International, 84, 29-36. (2001)

28. Quintana-Rizzo, J. Salter, R. Saul, S. Confirmation of AMphenicols in Honey using HPLC-Receptorgram. Apiacta, 38, 218-225. (2003)

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1. Saul SJ and Sugumaran M: Phenoloxidase activation, proteases and protease inhibitors in insect Hemolymph. Fed. Proc. Fed. Amer. Soc. Exptl. Biol. 44, 1860 (1986)

2. Saul SJ and Sugumaran M: Activation of two different phenoloxidases in the hemolymph of Sarcophaga bullata larvae in response to zymosan treatment.Proc. XVIII Int. Congr. Entomol. p. 143 (1988)

3. Sugumaran M., Rivera T, Semensi V and Saul SJ: Characterization of quinone methide generating cuticular phenoloxidase from Manduca sexta larvae.Proc. X-VIII Int. Congr. Entomol. p. 145 (1988)

4. Saul, SJ. Zomer, E and Charm SE: Charm Pesticide Test: Rapid Screening Method for the Detection of Organophosphate and Carbamate Pesticides for Water, Dairy Products, Fruits,Vegetables and Other Food Products. IAMFES. 80th Annual Meeting . 17-18, (1993)

Patents:

1. Zomer, E. Saul, S. and Charm, SE: Bioluminescence Method for the Determination of Pesticides.US Patent Number: 5,283,180. (1994)

2. Zomer, E. Saul, S. and Charm, SE: Method of Preparing D-Luciferin Derivatives, US Patent Number 5,374,534. (1994)

3. Zomer, E. Saul, S. and Charm, SE: Test kit for Determination of Organophosphate and Carbamate with Insect Brain Material That Hydrolyses a 6-Substitutes D-Luciferin Ester. US Patent Number 5,374,535. (1994)

4. Charm, SE. Skiffington, R. Markovsky, RJ. Zomer, E. and Saul SJ: Test Device for Detection of an Analyte. US patent Number 5,985,675. (1999) (note: see certificate of correction)

5. Markovsky, RJ. Boyer, CA. Charm SE. Donahue, PR. Glickman, YA, Saul, SJ. Scheemaker, JL. Skiffington, RT. Trivedi, ST and Zomer, E. Test Device for Detecting the Presence of a Residue Analyte in a Sample. US Patent Number 6,319,466. (2001)

6. Charm SE. Skiffington, R. Markovsky RJ. Zomer E. and Saul, SJ: Method for Detection of an Analyte. US Patent Number 6,475,805. (2002)

7. Zomer, E. Saul, S and Charm, SE; Test Kit and Method for the Determination of Pesticides. EP Patent Number 0576667. (2001)

and the time course of light emission was recorded. Using a luminometer it was also possible to detect the light emitted by intact bacteria in the presence of luciferin although the intensity of the light was greatly reduced in comparison to that emitted by the luciferase in cell extracts (data not shown). Presumably, this is due to a low permeability of the *E. coli* membrane to luciferin.

The smallest amount of firefly luciferase that we could detect on OG-1 X-ray film was 30 pg or 5×10^{-16} mol. This range of sensitivity could very likely be extended by using a high-speed film such as the ASA 20,000 Polaroid Land Type 612 instant film.[20] Much greater levels of sensitivity can be attained using a photomultiplier tube to detect the light emitted by the luciferase. One light unit is produced by 8.5 pg (1.4×10^{-16} mol) of luciferase when assayed in an Analytical Luminescence Laboratories Monolight 401 luminometer, and assay mixes lacking luciferase produced a background signal of 0.005 light units in the same instrument. A signal 10 times greater than the background was produced by 0.43 pg (7×10^{-18} mol) of firefly luciferase. The ease of performing assays for luciferase coupled with the great sensitivity of light detection systems make the firefly luciferase gene potentially very useful as a means of monitoring promoter activity in cells.

[20] G. H. G. Thorpe, T. P. Whitehead, R. Penn, and L. J. Kricka, *Clin. Chem.* 30, 807 (1984).

[2] Extraction of Adenosine Triphosphate from Microbial and Somatic Cells

By PHILIP E. STANLEY

There are many reasons for extracting and measuring adenosine triphosphate (ATP) from cells, but they may be placed into one of two main categories.

1. The level of endogenous adenosine triphosphate (ATP) in a cell may be used as an index of energy status. It is therefore useful in metabolic and physiological studies.[1]

2. Estimates of cell numbers in microbial and tissue cultures may be obtained after assuming that the ATP per cell remains at a fairly constant (within a factor of five) and known value under defined conditions. Thus by measuring total ATP in a sample of culture, cell numbers may be rapidly obtained. This is the basis of rapid microbiology using the ATP-firefly luminescence technique.[1-5]

[1] M. J. Harber, *in* "Clinical and Biochemical Luminescence" (L. J. Kricka and T. J. N. Carter, eds.), p. 189. Dekker, New York, 1982.

Extraction of ATP from cells and its subsequent measurement using the firefly luciferase procedure is often used. However there have been few studies to critically test the effectiveness of the adopted protocols for a wide range of cells.[6,7]

Properties of the Ideal Extractant

1. It should penetrate the cell wall and membrane more or less instantaneously.
2. It should extract ATP more or less instantaneously.
3. It should extract the target intracellular ATP pool completely.
4. It should instantaneously and irreversibly inactivate all enzymes that use ATP as a substrate or produce ATP from other substrates.
5. It should not cause breakdown of ATP (e.g., hydrolysis) either in short term (at the extraction time) or long term (during storage).
6. It should not have an inhibitory (quenching) effect on firefly luciferase during ATP assay.
7. It should not have an effect on the kinetics of firefly reaction.[8] Such an effect will cause problems of signal distortion and consequently internal standardization.
8. It should not extract undue quantities of extraneous materials which in themselves affect the firefly assay (by quenching or inhibition) and/or the result, e.g., colored agents, turbidity.[9]

In mixed cell populations, where, for example, bacteria and somatic cells exist, there may also be a need to selectively extract ATP from

[2] D. M. Karl, *Microbiol. Rev.* 44, 739 (1980).
[3] A. Lundin, *in* "Clinical and Biochemical Luminescence" (L. J. Kricka and T. J. N. Carter, eds.), p. 43. Dekker, New York, 1982.
[4] H. Van de Werf and W. Verstraete, *in* "Analytical Applications of Bioluminescence and Chemiluminescence" (L. J. Kricka, P. E. Stanley, G. H. G. Thorpe, and T. P. Whitehead, eds.), p. 33. Academic Press, London, 1984.
[5] D. Slawinska and J. Slawinski, *in* "Chemi- and Bioluminescence" (J. G. Burr, ed.), p. 533. Dekker, New York, 1985.
[6] A. Lundin and A. Thore, *Appl. Microbiol.* 30, 713 (1975).
[7] A. Lundin, *in* "Analytical Applications of Bioluminescence and Chemiluminescence" (L. J. Kricka, P. E. Stanley, G. H. G. Thorpe, and T. P. Whitehead, eds.), p. 491. Academic Press, London, 1984.
[8] L. J. Kricka and M. DeLuca, *Arch. Biochem. Biophys.* 217, 674 (1982).
[9] W. W. Nichols, G. D. W. Curtis, and H. H. Johnston, *Anal. Biochem.* 114, 433 (1981).

microbial or somatic cells. This imposes further constraints on the properties of the extractant.

When the sample itself is complex and perhaps variable, e.g., soil[10,11] or rumen contents,[12] this will put additional requirements on the properties of the extractant and extraction procedure.

It is worthwhile noting that properties 1 to 4 are associated with the extraction process alone whereas 5–8 have an affect on the ATP-firefly assay.

Note that properties 4 and 6 are apparently contradictory. However, the use of boiling buffer or cold acid (the latter requires immediate neutralization following extraction) does fulfill these needs but unfortunately also introduces sample dilution (and thus loss in sensitivity) or extra manipulation.

Treatment of Cells Which Affect ATP Level

Before extraction is performed it is usually necessary to take a sample; if this is not done properly ATP levels may change during this process. In addition a wide range of other factors have been shown to effect ATP levels[13] and the worker should be aware of these: (1) change of growth rate,[14] (2) change of nutient(s) or their concentration(s),[14–19] (3) change of gaseous environment, e.g., oxygen tension,[20,21] (4) change of temperature,[22] (5) change of pH,[23] (6) change of pressure,[24] and (7) change of light flux (for photosynthetic organisms).[17,25]

Laboratory techniques which one might employ to harvest or sample cells may well involve one or more of the above. I am thinking of centrifuging and filtration.

[10] J. M. Oades and D. S. Jenkinson, *Soil Biol. Biochem.* **11**, 201 (1979).
[11] J. J. Webster, G. J. Hampton, and F. R. Leach, *Soil Biol. Biochem.* **16**, 335 (1984).
[12] D. E. Nuzback, E. E. Bartley, S. M. Dennis, T. G. Nagaraja, S. J. Galitzer, and A. D. Dayton, *Appl. Environ. Microbiol.* **46** 533 (1983).
[13] P. C. T. Jones, *J. Theor. Biol.* **34**, 1 (1972).
[14] J. S. Franzen and S. B. Binkley, *J. Biol. Chem.* **236**, 515 (1961).
[15] L. Gustafsson, *Arch. Microbiol.* **120**, 15 (1979).
[16] M. Ohmori and A. Hattori, *Arch. Microbiol.* **117**, 17 (1978).
[17] A. Lewenstein and R. Bachofen, *Arch. Microbiol.* **116**, 169 (1978).
[18] C. M. M. Franco, J. E. Smith, and D. R. Berry, *J. Gen. Microbiol.* **130**, 2465 (1984).
[19] M. B. Nair and R. R. Eady, *J. Gen. Microbiol.* **130**, 3063 (1984).
[20] R. E. Strange, H. E. Wade, and F. A. Dark, *Nature (London)* **199**, 55 (1963).
[21] R. R. Mathis and O. R. Brown, *Biochem. Biophys. Acta* **440**, 723 (1976).
[22] Y. N. Lee and M. J. Colston, *J. Gen. Microbiol.* **131**, 3331 (1985).
[23] K. D. Beaman and J. D. Pollack, *J. Gen. Microbiol.* **129**, 3103 (1983).
[24] J. V. Landau, *Exp. Cell Res.* **23**, 539 (1961).
[25] P. C. T. Jones, *Cytobios* **6**, 89 (1970).

Therefore the experimental design should encompass a phase to ascertain whether or not the harvesting or sampling technique itself affects the ATP level.

Other Factors Which Affect ATP Level

These factors include the (1) age of cells or stage of growth,[23,26,27] (2) stage of cell division,[28–29] (3) density of cells,[30] (4) phage and virus[31–33] and microbial infections,[34] (5) action of agents which change cell type, e.g., tumor-promoting agents, and other agents such as (6) antibiotics[35,36] and certain drugs,[37] (7) metabolic inhibitors, cogeners of metabolites and toxins, (8) disinfectants,[38] pesticides,[39] and herbicides,[40] etc., (9) heavy metals, and (10) radiation, e.g., ultraviolet, microwave, X- and gamma rays.

General Considerations

A wide range of extractants have been used but few have been extensively investigated as far as effectivity. As well as those mentioned earlier[6,7] the group at NASA have published a series of reports[41–43] and at an

[26] K. W. Hutchison and R. S. Hansen, *J. Bacteriol.* **119**, 70 (1974).
[27] W. W. Forrest, *J. Bacteriol.* **90**, 1013 (1965).
[28] B. Chin and I. A. Bernstein, *J. Bacteriol.* **96**, 330 (1968).
[29] C. Edwards, M. Statham, and D. Lloyd, *J. Gen. Microbiol.* **88**, 141 (1975).
[30] L. Huzyk and D. J. Clark, *J. Bacteriol.* **108**, 74 (1971).
[31] I. Vlodavsky, M. Inbar, and L. Sachs, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1780 (1973).
[32] R. Wahl and L. M. Kozloff, *J. Biol. Chem.* **237**, 1953 (1962).
[33] E. Egberts, P. B. Hackett, and P. Traub, *J. Virol.* **22**, 591 (1977).
[34] Y. Tsuchiya and H. Sugai, *Biochem. Med.* **28**, 256 (1982).
[35] P. McWalter, *J. Appl. Bacteriol.* **56**, 145 (1984).
[36] R. Guerrero, M. Llagostera, A. Villaverde, and J. Barbé, *J. Gen. Microbiol.* **130**, 2247 (1984).
[37] G. E. Thomas, S. Levitskyy, and H. Feinberg, *J. Mol. Cell Cardiol.* **15**, 621 (1983).
[38] J. E. Cairns, S. G. Nutt, and B. K. Afghan, *in* "International Symposium on Analytical Applications of Bioluminescence and Chemiluminescence" (E. Schram and P. Stanley, eds.), p. 303. State Printing and Publishing, Westlake Village, California 91361, 1979.
[39] R. D. Gruenhagen and D. E. Morelanď, *Weed Science* **119**, 319 (1971).
[40] T. J. Clegg and J. L. Koevenig, *Bot. Gaz.* **135**, 368 (1974).
[41] E. A. Knust, E. W. Chappelle, and G. L. Picciolo, *in* "Analytical Applications of Bioluminescence and Chemiluminescence," p. 27 (and other references). NASA Document SP-388, 1975.
[42] G. L. Picciolo *et al.*, *in* "Applications of Luminescence Systems to Infectious Disease Methodology." Goddard Space Flight Center, Greenbelt Maryland, Document X-726-76-212, 1976.
[43] E. W. Chappelle, G. L. Picciolo, and J. W. Deming, this series, Vol. 57, p. 65.

early date highlighted the fact that bacterial ATP turns over rather quickly.[44] A number of comparative studies have been made for environmental samples (marine waters, etc.).[45-47].

In general, many bacteria have ATP levels around 1 fg (10^{-15} g) per cell. Yeasts have around 100 times more and many animal cells contain around a picogram ATP (10^{-12} g). Some actual values are given in refs. 1–3.

I will now consider, albeit briefly, the main extractants and give a few references wherein details can be obtained.

1. Boiling buffer, usually Tris–Cl, with EDTA; marine microbial samples,[2] freshwater microbial samples,[48] nematodes,[49] yeast,[50] rumen contents,[12] mycoplasmas,[23] mycobacteria.[22]
2. Various dilute acids including nitric acid (mainly clinical samples,[41-43] sulfuric acid [seawater samples,[2,45,47] clinical samples (ref. 47, p. 189)], perchloric acid (microbial samples,[14,27,51] erythrocytes,[34] tumor,[33] plants[52]), trichloroacetic acid (microbial samples,[6,7] somatic cells[3,7]), and formic acid (microbial samples[44]).
3. Organic compounds including dimethyl sulfoxide (bacteria),[26] ethanol (alga),[53] acetone (yeast),[54] chloroform (mycobacteria),[55,56] and butanol (bacteria).[57]
4. Surfactants including Triton X-100 (somatic cells)[3,6,7] and benzalkonium chloride (yeast).[58]

In addition various mixtures have been described which include some of those extractants mentioned above and which have been used for com-

[44] W. Klofat, G. Picciolo, E. W. Chappelle, and E. Freese, *J. Biol. Chem.* 244, 3270 (1969).
[45] O. Holm-Hansen and D. M. Karl, this series, Vol. 57, p. 73.
[46] "ATP Methodology Seminar" (G. A. Borun, ed.). SAI Technology Co., San Diego, California, 1975.
[47] "2nd Bi-Annual ATP Methodology Symposium" (G. A. Borun, ed.). SAI Technology Co., San Diego, California, 1977.
[48] B. R. Taylor and J. C. Roff, *Freshwater Biol.* 14, 195 (1984).
[49] H. J. Atkinson and A. J. Ballantyne, *Ann. Appl. Biol.* 87, 167 (1977).
[50] A. Cockayne and F. C. Odds, *J. Gen. Microbiol.* 130, 465 (1984).
[51] M. Statham and D. Langton, *Process Biochem.* 10, Oct. 25 (1975).
[52] P. E. Stanley, *in* "Liquid Scintillation Counting" (M. A. Crook and P. Johnson, eds.), Vol. 3, p. 253. Heyden and Son, London, 1974.
[53] J. B. St John, *Anal. Biochem.* 37, 409 (1970).
[54] L. F. Miller, M. S. Mabee, H. S. Gress, and N. O. Jangaard, *J. Am. Soc. Brew. Chem.* 36, 59 (1978).
[55] A. M. Dhople and E. E. Storrs, *Int. J. Leprosy* 50, 83 (1982).
[56] R. P. Prioli, A. Tunna, and I. N. Brown, *Tubercle* 66, 99 (1985).
[57] E. C. Tiffi, Jr., and S. J. Spiegel, *Environ. Sci. Technol.* 10, 1268 (1976).
[58] M.-R. Siro, H. Romar, and T. Lovgren, *Eur. J. Appl. Microbiol. Biotechnol.* 15, 258 (1982).

plex samples such as soil.[10,11] Further a number of commercial extractants of undisclosed content are available but little has been published about their efficiency in comparison with those listed above.[4,56]

In all cases it is important not to overload the extractant with too much sample. Usually a few milligrams dry weight of sample per milliliter of extractant is satisfactory. It is also important to check the sampling/extraction procedure to ascertain that the process itself does not change the ATP level or influence the measured result. An example of poor sampling might be in drawing a blood sample from an animal and allowing the sample to hemolyze, thereby releasing ATP into the plasma. Another might be to allow a microbial sample to become nutrient deficient during sampling.

As mentioned previously there have been few critical studies in this area. One of the first[6] involved the use of 5 microbial species with 10 different extractants. From this study, the workers concluded that only trichloroacetic acid (TCA) was entirely satisfactory for all species. In a follow up,[7] 5 different extractant systems were tested on 7 species of microorganisms and 9 different types of somatic cells. From both studies it was concluded that trichloroacetic acid should be the standard against which other extractants should be tested. In the second of the studies the authors suggest 10, 5, 2.5, and 1.25% TCA (final concentration: equal volumes of TCA and sample) should be employed initially as a standard before searching for better extractants of ATP. In coming to a decision, the total luminescence as well as the lowest degree of quenching of luciferase by the extractant need to be taken into account when choosing operational parameters. It is not possible to cover all types of extractants and their use in a wide range of cells. I will therefore indicate some guidelines to follow when designing extraction protocols.

Somatic Cells

Cells which are separate from one another (e.g., blood cells, certain tissue cultures) do not generally need special treatment prior to extraction and can be mixed directly with the extractant. An exception would be a case in which extracellular (free) ATP was present in amounts that could not be neglected and here cells would need washing or the ATP removed enzymically. However, cells that are formed together in a more or less solid tissue from living organisms (e.g., muscle, kidney, plant leaves) should be frozen to stop the action of ATPases and then homogenized or thinly sliced (<0.2 mm). Freezing is best done in liquid nitrogen and homogenizing with a blender or a simple mortar and pestle. Slicing can be done with a razor blade or on a cryomicrotome.

If small amounts of ATP are expected it is important that equipment be kept scupulously clean and free from microbial contamination. Follow-

ing homogenization or slicing, an aliquot of the sample can then be allowed to thaw in the presence of the extractant so long as they are well mixed and no clumps of tissue are formed. It is essential that extractant be quickly and intimately associated with all cells. If the cells clump together the extractant may take a long time to penetrate to the inner cells by which time considerable changes in ATP content may have taken place. Alternatively the final result may be too low because little or no ATP has been extracted from those cells. The problem of clumping may be solved in some cases by subjecting the extracting sample to ultrasound.

Alternatively tissue samples may be homogenized at ice water temperature in a glass–glass homogenizer, blender, etc. Another approach would be to homogenize the sample in boiling buffer (e.g., nematodes[49]). For large samples of tissue, 10 g tissue in 100 ml buffer may be homogenized as described in ref. 59 at room temperature or in a cold room.

Microbial Cells

Bacterial cultures can often be added directly to the extractant. For acidic extractants 1 ml culture to 1 ml acid is generally satisfactory and if boiling buffer is used then 1 ml culture into 10 ml boiling buffer should be employed.

Fungal cultures with mycelia and algal cultures with multicellular filaments may require homogenizing in a blender in the presence of the extractant.[60]

Because yeast cells and microbial spores have thicker walls than bacteria they consequently often require more drastic extractants or a longer extraction time.

When yeast and bacteria are both present in the sample (e.g., urine) one method of measuring both is to use a differential filtration procedure.[61] I am not aware of any extraction procedures which permit differentiation of two or more microbial species in the same sample without complex manipulation, e.g., cell separation or growth in selective media.

When microbial numbers are small a concentration step may be required and filtration or centrifuging may be convenient. Both methods tend to lead to a decrease in ATP content because of oxygen and nutrient depletion. The problem can be usually solved by adding a small volume of a suitable culture medium to the filter or pellet and allowing the microbial cells to recover for 5–10 min before extracting them.

[59] C. J. Stannard and P. A. Gibbs, *J. Biolumin. Chemilumin.* **1**, 1 (1986).
[60] N. A. Hendy and P. P. Gray, *Biotechnol. Bioeng.* **21**, 153 (1979).
[61] T. S. Tsai and L. J. Everett, *in* "Analytical Applications of Bioluminescence and Chemiluminescence" (L. J. Kricka, P. E. Stanley, G. H. G. Thorpe, and T. P. Whitehead, eds.), p. 75. Academic Press, London, 1984.

Somatic and Microbial Cells Mixed Together with Nonliving Material

This is the most complex system. Examples in this section include soil,[4] nematodes in soil,[49] meat,[59] milk,[62] rumen contents,[12] fruit juices,[63] and clinical samples.[1,3,42,43]

Suppose you wish to determine microbial ATP in one of the above samples. In most situations the somatic ATP will be present in large excess (perhaps 10^6-fold) and this can pose considerable technical problems, especially if a number of samples are to be processed. Two approaches have been used. In the first, somatic cells are selectively extracted (e.g., using Triton X-100) and the somatic ATP together with any free ATP can then be hydrolyzed with an ATPase.[43] The second method involves physical separation of somatic and microbial cells by differential centrifuging or filtration or addition of resin.[59] In both cases the remaining microbes can then be extracted.

As far as differential extraction is concerned use is generally made of the substantial difference in cell wall/membrane in microbial and somatic cells. Care however must be exercised since so-called somatic extractants (e.g., Triton X-100) have been shown to extract ATP from some bacteria (e.g., *Pseudomonas aeruginosa*.)[7] Microbial extractants will in most cases also extract ATP from somatic cells.

There are two other sources of ATP in many samples. First, free ATP, that is ATP which is in true solution. This will generally be measured and not distinguished from cellular ATP unless it is removed enzymically or by washing. Second, there is the more problematic "bound" ATP which is sequestered on protein and other macromolecules and surfaces in a fairly firm fashion. Bringing the pH of the sample below 4.5 using malic acid has been used to release this ATP so that intereference by it can be removed.[43]

Some Other Points Concerning Extractants

The use of boiling buffer is popular because of simplicity and is quite satisfactory if very high sensitivity is not required. If sensitivity is a requirement then the dilution involved (usually 10-fold) is a disadvantage. Be sure to have the buffer boiling before addition of sample (at least 1 : 5, or better 1 : 10 sample : buffer) so that enzyme denaturation is immediate. If it is not ATPases and other enzymes will change the ATP content even if they are active for say 10 sec.

It is best to avoid as much as possible any postextraction processing as this inevitably leads to losses of ATP during manipulation and to prob-

[62] R. Bossuyt, *Milchwissenschaft* **36**, 257 (1981).
[63] J. G. H. M. Vossen and H. D. K. J. Vanstaen, *Forum Mikrobiol.* **4**, 280 (1981).

lems of contamination because of the ubiquitous nature of ATP (e.g., in sweat, contaminating microbes, etc.) which may be a problem if you are working at high sensitivity.

The use of perchloric acid has led to two reports which indicate other types of problems. Instead of adding the perchloric acid extract directly to the firefly assay system as is usually the case it is possible to reduce the quenching caused by the acid if one removes the acid by precipitating it as the insoluble potassium salt and finishing with a sample at neutral pH. However, one report indicates that some ATP coprecipitates with potassium perchlorate so that final ATP measurements are too low.[64] Another set of workers have reported that on neutralizing perchloric acid extracts from *Bacillus brevis* a phosphatase is reactivated which then proceeds to hydrolyze extracted ATP![65]

If organic solvents are used, some of them may be readily evaporated and this provides an easy way to increase sensitivity should that be necessary.[55]

[64] S. Wiener, R. Wiener, M. Urivetzky, and E. Meilman, *Anal. Biochem.* 59, 489 (1974).
[65] J. A. Davison and G. H. Fynn, *Anal. Biochem.* 58, 632 (1974).

[3] Detection of Bacteriurea by Bioluminescence

By BRUCE A. HANNA

Urinary Tract Infection

Microbial colonization of the urogenital tract, resulting in bacteriurea, is an increasingly common event in modern medical management. While normally sterile, the urinary tract which includes the kidneys, ureters, urinary bladder, and urethra may readily become infected with a wide variety of microbes. Frequently, such infections are preceded by instrumentation and manipulation of the urinary tract, often by insertion of a urinary catheter. As a result, normal flora microbes, particularly skin and enteric bacteria, may gain entry to urinary tract tissues. The severity of such infections may range from those which are asymptomatic except for the presence of bacteriurea, to overt clinical infections of the bladder (cystitis) or kidney (pyelonephritis) which may be accompanied by severe systemic symptoms. Evaluation of the urine to determine the presence and concentration of microorganisms in the urine is an important adjunct in the diagnosis and treatment of urinary tract infections.

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Significant Bacteriurea

The definition of what represents clinically significant bacteriurea has been the subject of much discussion. In actuality, the criteria for defining significant bacteriurea is dependent on the patient from whom the sample is derived.[1] The conventional criterion invoked to detect urinary tract infections in asymptomatic patients where there is a low prevalence of disease in the population, is 10^4–10^5 colony-forming units (CFU)/ml in a freshly voided, first morning specimen.[2] In this select patient population counts of $>10^5$ are almost certainly significant, while counts of $<10^4$ have a high probability of representing urethral contamination. In symptomatic patients, on the other hand, where the prevalance of urinary tract infection in the population is high, colony counts of 10^3 or even 10^2 CFU/ml may be considered significant.[3] Frequently in such patients the urine will contain numerous polymorphonuclear leukocytes and other blood cell components as well as bacteria. In addition to bacteria, urinary tract infections may on occasion be caused by fungi, especially *Candida* species, *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Mycobacteria* and viruses.

Detection of Bacteriurea

The techniques available to determine the presence and quantity of microorganisms in a urine specimen can be divided into those that are growth dependent and those that are non-growth dependent. Growth-dependent methods, by definition, require dilution, inoculation onto a suitable medium, and an incubation period of 18–24 hr. This will result in an enumeration as well as a prelude to the identification of the microbes present. Non-growth-dependent methods, in contrast, do not require cultivation of the organism, but rather provide a direct enumeration of the bacterial population present. Since the noncultivation methods provide the user with a quantitation but not an identification. they are termed screening tests.

In a typical clinical microbiology laboratory as many as 70% or more of urine specimens may not contain significant populations of bacteria. In such a setting, bacteriurea screening tests are very useful in eliminating these samples from further analysis. Intrinsic to these methods is their ability to rapidly identify such samples on the same day as they are collected from the patient. Conversely, as the majority of urinary tract

[1] R. C. Bartlett and R. C. Galen, *Am. J. Clin. Pathol.* 79, 756 (1983).
[2] R. Plott, *Am. J. Med.* 75(1B), 44 (1983).
[3] W. E. Stamm, G. W. Counts, K. R. Running, S. Fihn, M. Turck, and K. K. Holmes, *Am. J. Med.* 307, 463 (1982).

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Published
With international search report.
With amended claims.

(54) Title: ATP EXTRACTION REAGENT CONTAINING AMMONIUM VANADATE

(57) Abstract

An Ammonium Vanadate containing ATP extract reagent for extracting ATP from somatic tumor cells without disruption of the cell membrane from 2.6-4.6 millimolar of Ammonium Vanadate stabilizes the extracted ATP. Employment of the Ammonium Vanadate containing ATP extract reagent in the bioluminescent reaction method for assaying the effectiveness of chemotherapeutic agents against disassociated tumor cells constitutes an overall improvement in the method.

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ATP Extraction Reagent Containing Ammonium Vanadate

This invention relates to an ATP releasing reagent and in a preferred embodiment relates to an ATP releasing reagent adapted for employment in a bioluminescent assays of somatic tumor cells through measurements made of ATP extracted from the cells.

This invention also relates to an improved method for screening tumor cells to ascertain their sensitivity to chemotherapeutic agents.

BACKGROUND OF THE INVENTION

The selective determination of nucleotides in a sample through the firefly bioluminescent measurement of the adenosine triphosphate (ATP) content in a sample is a rapid and sensitive method for ascertaining the viability and number of cells in the sample. This bioluminescent measurement has been applied in the past to analytical test measurements that determine the number and viability of bacterial cells in a test sample, see U.S. Patent No. 3,745,090 and more recently to test measurements for the number and viability of either or both of somatic and microbial cells in a test sample, see U.S. Patent No. 4,303,752.

In vitro analytical tests on cellular ATP employing the firefly luminescent reaction with ATP have become sufficiently popular to engender commercial availability of ATP releasing reagents from laboratory supply houses as, e.g., FL-SAR™ (Sigma Chemical Co.), SOMALIGHT™ (Luminescence Analytical Laboratory) and PICOEX™ (Packard Instrument Co.). To conduct an assay the somatic cell sample is suspended in a solution containing an ATP releasing reagent composition, and

then the ATP content of the solution is measured through the firefly luminescence reaction.

In common, these ATP release reagents include a (non-toxic) wetting agent, e.g., non-ionic surface agents whose purpose is to render the cell membrane permeable to whatever relatively small molecules are present among the (cellular) constituents inside of the cell membrane. Thereafter, the small molecules diffuse out past the cell membrane barrier. In particular, ATP diffuses out past the cell membrane barrier so that the ATP content still inside of the cells and the ATP content in the aqueous medium surrounding the cells approaches an equilibrium that is in proportion to the number of (viable) cells suspended in the medium. Preferred non-ionic surface agents are the ethoxylated phenols and the fatty acid polygycol ethers. A more detailed discussion of the non-ionic surface active agents preferred for the ATP releasing reagent purposes with somatic cells and with microbial cells can be found in the U.S. Patent No. 4,303,752 to which reference is made. Preferred for the surface active agent are TRITON-X or NONIDENT P-40. It is important to note that the surface active agent is present in relatively low proportions, say 0.5 - 0.1%. In practice of this invention as in practice of 4,303,752 release of the ATP is accomplished without complete destruction of the cell membrane of the viable cells. Complete destruction of the cell membrane may release large molecular weight cellular constituents from inside of the cell including notable enzymes which would act on ATP.

In common, these ATP release reagent compositions known to the art also include a buffer composition whose purpose is to adjust the pH of the cell suspension in the (diluted) release reagent to some desired level. In the instance of ATP release reagents for somatic cells, any of the usual buffers,

e.g., tris may be employed but Hepes at pH 7.7 - pH 7.9 is a preferred buffer. Hepes which is N-[2-hydroxy-ethyl]piperazine N' -[2-ethanesulfonic acid] does not interfere with the firefly bioluminescent reaction measurement of the ATP content in the buffered solution and, desirably, is adapted to buffer the suspension at a pH of about 7.8, which is an optimum level for the firefly bioluminescent reaction.

A variety of in vitro assay screening methods have been developed over the past two decades to test the applicability of chemotherapeutic drugs against tumors. The method for ascertaining the sensitivity of tumor cells to chemotherapeutic agents to which the practice of this invention pertains, comprises culturing a multiplicity of samples of tumor cells in the presence of different chemotherapeutic drugs at varying concentrations and then assaying the proportion of tumor cells that remained viable after 4-7 days in vitro using the firefly bioluminescent reaction for the assay. Ascertained thereby is the affect of different concentrations of the therapeutic drugs on the tumor cells. For the details of how this screening method has been conducted heretofore reference is made to Sevin et al., "Application of an ATP-Bioluminescence Assay in Human Tumor Chemosensitivity Testing", Gynecol. Oncol., 31:191-204 (1988). However, Sevin et al. disrupt the tumor cells. In the assay method of the present invention the cell membranes of the somatic tumor cells are not destroyed and, moreover, the firefly bioluminescent reaction is carried out on stabilized ATP (in solution).

BRIEF DESCRIPTION OF THE INVENTION

Briefly stated, the ATP release reagent of the present invention comprises a solution of known in the art components for ATP release reagents in art recognized proportions to render cell membranes

permeable without destruction of the cell membrane. Also present is NH_4VO_3 (Ammonium meta Vanadate) in proportions effective to inhibit any cellular ATPase enzyme(s) that might otherwise react away extracted ATP in the solution. In the absence of Ammonium Vanadate the level of ATP in the suspension of somatic tumor cells in the ATP release reagent composition rapidly decreases.

The range of concentrations of Ammonium Vanadate contemplated for the ATP release reagent composition of this invention is about 8-14 mM so that a proper concentration of the Ammonium meta Vanadate preferably in the range of about 2.6 to 4.6 mM (millimolar) appears in the cell suspension.

A separate aspect of this invention is an improved assay method for determining the sensitivity of tumor cells to chemotherapeutic agents, the improvement features comprising incorporating Ammonium Vanadate in ATP release reagent compositions that are adapted to release ATP from human tumor (somatic) cells without totally disrupting the cell membranes.

RATIONALE OF THE INVENTION

According to the method for assaying the sensitivity of tumor cells to chemotherapeutic agents described by Sevin et al. supra, extracting ATP from the tumor cells is done by a cell lysis technique using a strong acid such as trichloroacetic acid (TCA). However, lysis of the tumor cells releases ATPase enzymes from the cell into the ATP extract solution. Also this procedure requires another buffer reagent to achieve pH 7.8. Extracting ATP from viable cells according to practice of U.S. Patent No. 4,303,752 with ATP release reagents which do not totally disrupt the (tumor) cell membrane is believed to be a more advantageous method of securing an ATP content in solution that is in proportion to the viable cell count

of the sample, and such is done in the method of this invention.

In the instance of human tumor cells, it has been found, however, that the ATP extracted according to the technique of U.S. Patent No. 4,303,752 becomes eliminated rapidly from the ATP release reagent solution. Indeed ATP is not readily detectable in the somatic cell suspension release reagent solution within only about 15 minutes after extraction of the ATP into the ATP release reagent composition. It is believed that cellular ATPase enzyme(s) degrade the ATP. However, when effective amounts of Ammonium Vanadate have been included in the ATP release reagent, the ATP extracted into the cell suspension remains relatively stable. Measurements of extracted ATP can be obtained reproducibly for up to about four hours after the ATP extraction step.

Ammonium Vanadate seems to have a molecular structure similar to ATP, and apparently the Ammonium Vanadate binds with ATPase enzymes at an active site thereon. Whatever the reaction mechanism may be, Ammonium Vanadate inhibits the catalytic activity of ATPase enzymes on ATP, an inhibition which is known in the art, see Cantley et al., J. Bio. Chem., 252:7421 (1977).

The great advantage to stabilizing the ATP which has diffused from the tumor cells into solution in the ATP release reagent should be self-evident. The assay method of this invention is adapted to an essentially concurrent evaluation of a great many tumor cell samples so as to test varying amounts of different chemotherapeutic agents on the tumor. Preferably extraction is performed in all samples. Then the ATP in the samples are measured one by one. Stabilizing the ATP in each sample allows time for extraction and measuring the ATP from tumor cells from each sample, in proportion to the number of cells in the sample,

despite the widely differing tumor cells content in some of the multitude of samples. In addition, stabilizing the ATP content allows the time needed to manipulate the multitude of samples however necessary up to introduction of the luciferase-luciferin reagents into each sample, one by one for conduct of the firefly luminescence reaction assay. For carrying out the comprehensive assay method of this invention on human tumor cells, the ATP in solution in the particular tumor samples measured by the firefly bioluminescence reaction should be sufficiently stable to allow up to about 2.5 hours of delay between the addition of the NH_4VO_3 extraction reagent into the cell suspension medium and conduct of the firefly bioluminescent reaction.

DETAILED DISCUSSION OF THE INVENTION

A principal aspect of the invention is the presence of effective amounts of NH_4VO_3 in the ATP releast reagent. The Ammonium Vanadate content is in the range of about 2-6-4-6 millimolar and preferably in the range of about 3.0-4.0 millimolar for achieving an optimum stability of ATP counts. At concentrations about about 4.0 millimolar, a discernible decrease in counts has been observed. This is believed to be due to inhibition of the luciferase enzyme caused by excess NH4VO3 in the extracted sample. As expected, counts decrease as a function of decreasing NH_4VO_3 concentration and such occurs below the 3.6 millimolar concentration in the tumor cell suspension. The release reagent of this invention is particularly adapted for the release of ATP from human tumor cells without total disruption of the tumor cell membranes.

As already has been indicated herein, a second aspect of the present invention resides in an improved method for evaluating the sensitivity of somatic tumor cells to chemotherapeutic agents. The

early steps in the process generally follow the procedures described by Sevin et al., supra, but the final steps in this process involved extracting ATP from the cultured somatic tumor cells without totally disrupting the cell membrane into an NH_4VO_3 containing (ATP release reagent) solution, after which the solution is assayed for its ATP content by the firefly bioluminescence reaction.

Overall, the use of the ATP bioluminescence reaction to measure the effect of chemotherapeutic drugs against tumor cells cultured in vitro has been so simplified by practice of this invention, that tumor chemosensitivity assays can be performed rapidly and easily in a standardized format. In addition, the improved method of this invention provides an objective measurement determinative of sensitivity of the tumor cells to various drugs, using relatively few tumor cells in each sample.

BRIEF DESCRIPTION OF THE DRAWINGS

The assay method of this invention will now be described in relation to the attached drawings wherein:

Figure 1 is a flow sheet representation of the method;

Figure 2 is a graph showing luminometer counts 15 minutes after extraction; and

Figure 3 is a graph showing luminometer counts 30 minutes after extraction.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

Referring now to Figure 1 of the drawing, it may be seen that the first step of the method transforms a solid tumor specimen into a suspension of single cells or multi-cell aggregates of less than about 30 cells per aggregate. In the illustrated preferred mode of this invention, this transformation

is performed as described by Sevin et al., supra. First, the solid tumor specimen is minced into 1-5 mm pieces. Then, the minced tumor pieces are disassociated enzymatically into single cells and small size multi-cell aggregates by dispersing about 1 gram of minced tumor in sterile culture medium to which had been added about 1500 units per ml of DNase 1, about 2 mg/ml of collaganase and about 1 mg/ml of Dispase enzymes. Use of this particular combination of enzymes is a known in the art technique for disassociating solid tumor cell pieces into individual cells and (tiny) cell aggregates containing less than about 30 cells per aggregate.

The second step of the method comprises culturing a multiplicity of samples of the disassociated tumor cells in a standard nutrient medium for from 4-7 days. The growth medium and cultivation conditions is suitably described by Sevin et al. (McCoy's enriched media with 15% fetal calf serum at 37°C at 95% humidity for 6-7 days) in the presence, sample to sample, of varying concentrations of different chemotherapeutic agents. Of course, an appropriate number of control samples of the tumor cells are also cultured. One special consideration involved in culturing the disassociated tumor cells in the presence of chemotherapeutic drugs is that frequently normal cells will be present along with the tumor cells in some or all of the samples. Normal cells are an interfering substance in the assay method. ATP measurements derived from a normal cell's content in the samples are not desired. To the extent reasonably possible, normal cells should be eliminated from the test samples of tumor cells.

Fortuitously, normal cells are generally anchorage dependent cells. Therefore, culturing mixtures of disassociated normal and tumor cells inside of sample wells provided with wall surfaces to which

normal cells cannot anchor, will prevent proliferation of whatever normal cells are in the samples while allowing proliferation of the (non-anchorage dependent) tumor cells. Hopefully, all normal cells will die over the course of culturing period, while a significant number of tumor cells remain viable at expiration of the culture period. A preferred embodiment culture period is 4-7 days.

Agarose coated microtiter plates prepared as described in copending application, S.N. 07/651,940, filed February 7, 1991, is preferred for culturing the tumor cell samples. The agarose coated well bottom will prevent proliferation of normal cells therein. Of course, other techniques for placing an agarose coating on the well bottom of the commercially available polystyrene microtiter plate than through practice according to S.N. 07/651,940 may be used. Alternatively, polypropylene microtiter plates may be employed for culturing the tumor cell samples according to the method of this invention.

The third step in the bioluminescent method of this invention is extraction of the tumor cell cultures (without total disruption of the cell membrane) so as to release ATP from the tumor cells in proportion to the number of viable tumor cells in the culture. The resulting ATP solution is stabilized by the presence of Ammonium Vanadate in the solution. Other than the presence of Ammonium Vanadate therein, the ATP releasing reagent is comprised of known in the art ingredients. For example, one of the commercially available ATP release reagents FL-SAR™, SOMALIGHT™ or PICOEX™ may have a concentrated solution of Ammonium Vanadate added thereto generate 2.6-4.6 millimolar NH_4VO_3 in the cell suspension medium and then the solution is used to release ATP from the somatic tumor cell suspension following the details of the procedure set out in U.S. Patent No. 4,303,752. As a point of

preference, the ATP release reagent has been buffered with 0.1 - 0.35 M Hepes which is a relatively high buffer content. Typically, the pH of the tumor cells sample drops during cultivation to about pH 6.5, and the final pH (for the bioluminescent reaction) is pH 7.7 - 7.9. Preferably, the Hepes concentration in the cell suspension is about 0.125 M.

In the test study which is illustrated in Figures 2 and 3, the Ammonium Vanadate dissolved in distilled water was mixed with a 0.35 M Hepes solution and either NP-40 or Triton X-100 to 0.03-0.50% thereof by wt was incorporated to form the ATP releasing reagent. Typically, two parts of tumor cell suspension is mixed with one part of the ATP releasing reagent.

About 0.15% by wt of Triton X-100 or NP-40 seems to be a reasonable optimum and such constitute preferred embodiment wetting agents and the content thereof.

For conducting mass screening of therapeutic agents for effectiveness against tumors by the method of this invention it is important that the ATP extracted into the ATP release reagent should be relatively stable and not be degraded by ATPase enzymes indigenous to the tumor cells. Stabilizing the ATP dissolved in the medium in which the cells are suspended allows time to assay a great number of samples routinely. Desirably, the ATP content in the cell suspension medium should be stable enough to allow for quantitative reproductability of the ATP measurements made with the firefly bioluminescence reaction throughout about a 2 1/2 hour period following extraction of the ATP from each of the cultured tumor cell samples. Thus, stabilization of the ATP in solution in the cell suspension medium extract should solution through inclusion of Ammonium Vanadate in the ATP release reagent is important to practice of the overall test assay method of this invention.

For further understanding of this invention the following specific examples are provided.

Example 1

ATP extraction reagents containing 0.32 M Hepes buffer pH 7.8, varying amounts of Triton X-100 or NP-40 in the range of from 0.5 to 0.03% by wt with and without the inclusion of 10.86 mM NH_4VO_3 in the reagent were prepared and then used to extract ATP from ME180 ovarian tumor cells. The tumor cell extracts were counted by conducting the firefly bioluminescence reaction on Sample 15, 30 and 60 minutes after the ME180 tumor cells suspended in a fetal calf serum culture medium were first mixed with the ATP extraction reagent. In the absence of Ammonium Vanadate the ATP extracted from the tumor cells became rapidly eliminated from the reagent and little ATP was detected after only about 15 minutes, whereas good stability of the ATP counts for more than 60 minutes was evidenced when Ammonium Vanadate was present.

The results of the 15 and 30 minute study are provided in Figures 2 and 3. The rapid disappearance of ATP from unstabilized solutions is evidenced. The data shows that best ATP extraction is obtained with 0.13% by wt or more of the wetting agent.

Similar results have been obtained with other tumor cell lines, e.g., SW-948 colon and with tumors secured from patients. In all of the studies, the extracted ATP was stabilized by the presence of Ammonium Vanadate in the ATP extraction reagent.

It is believed that the optimum cell concentrations for being assayed with the preferred tumor cell extraction reagent is 10,000-80,000 cells/well of a 96 well flat bottom microwell plate in approximately 200 μl of culture media per sample.

Example 2

To carry out the comprehensive method of this invention a solid tumor specimen is minced and enzymatically disassociated as described in Sevin et al., supra, to which reference is made for details of the work up procedure. Briefly, the work up procedure is to disassociate the solid tumor as is illustrated in Figure 1 herein by mincing the same while the tumor cells are being bathed in a nutrient medium containing fetal calf serum, sufficient antibiotic to control possible microbial contamination and a tumor disassociation enzyme as already has been described herein. Then the minced tumor pieces are enzymatically cultured in more of the same nutrient solution mixture at 37°C until the tumor cells have visually been disassociated or as convenient, say for 8-16 hours.

Optionally, but preferably, ficoll-hypaque density gradient separation is used to reduce dead cell and erythrocyte contamination.

The disassociated tumor cells are washed, then resuspended in the fetal calf serum nutrient medium at a cell concentration of about $4x10^5$ cells per ml. About 0.1 ml of this suspension is added to each well of a 96 well agarose coated polystyrene culture plate. Parenthetically, it is noted that the best range of tumor cell concentration per well is from $2x10^4$ - $8x10^4$.

An essay for later in vivo treatment of the tumor is conducted by testing each proposed chemotherapeutic drug at the four concentrations corresponding to 12.5%, 25.0%, 50.0% and 100% of the estimated Peak Plasma Concentration (PPC) for the drug using triplicate cultures for each drug concentration. Fourteen chemotherapeutic drugs can be tested on two 96 microwell culture plates. The wells of the culture plates which remain open are used for MO "No Drug" and MI "Maximum Inhibitor" controls. Alternatively, each

proposed chemotherapeutic drug may be tested at eight concentrations, the additional concentrations being 1.56%, 3.13%, 6.25% and 200%. In this event, only ten chemotherapeutic drugs can be tested on two 96 microwell culture plates.

The drugs in appropriate concentration dissolved in the fetal calf serum nutrient medium are applied from a multi-channel pipette to the wells in the culture plates and then the microwell culture plates containing 0.2 ml per well of the drug dosed tumor cell culture samples are incubated for seven days in a humidified, 37°C, 5% CO_2 incubator. If rapid cell proliferation is evidenced, the incubation period should be decreased to 4-6 days.

The Ammonium Vanadate stabilized tumor cell extraction reagent formulated as described herein, is then added to each well in the culture plate at the rate of 0.1 ml per well and thoroughly mixed in to form a cell suspension in the mixture of culture medium and ATP extraction reagent that constitutes a Hepes buffered 0.05% by wt of Triton X-100, 3.62 mM NH_4VO_3 stabilized solution of ATP. The suspension is incubated about 20 minutes at room temperature before an aliquot thereof and the luciferin-luciferase counting reagents are mixed for measuring the ATP content. Parenthetically, it is noted that best results are obtained if the cell cultures are biolumimetrically counted within 60 minutes of the addition of tumor cell extraction reagent.

For actual counting of the ATP, .05 ml aliquots of the extracted tumor cell cultures are transferred into tubes and .05 ml per well of a known to the art luciferin-luciferase counting reagent for ATP measurement is injected into the aliquots one by one and the bioluminescent counts are measured over a 20 second period in a luminometer (a count integration time in the range of 15-30 seconds is recommended).

CALCULATION AND INTERPRETATION OF LUMINOMETRY RESULTS

The percentage of tumor cell growth inhibition for each test drug concentration is calculated by the equation:

$$1.0 - \frac{(TEST) - (MI)}{(MO) - (MI)} \times 100 = \text{Percent Inhibition}$$

(TEST) = Mean counts for test drug cultures

(MI) = Mean counts for Maximum Inhibitor controls

(MO) = Mean counts for No Drug controls

STRONGLY SENSITIVE: Mean inhibition for 3.13% - 25% PPC drug concentrations is 70% or greater. Associated with a 60-70% probability of a favorable response in vivo.

PARTIALLY SENSITIVE: Mean inhibition for 3.13% - 25% PPC drug concentrations is 50% - 70%. Associated with a low probability of a favorable response in vivo.

RESISTANT or STIMULATORY: Mean inhibition for 6.25% - 25% PPC drug concentrations is less than 50%. Inhibition of less than 15% may reflect a stimulatory effect of the test drug.

An assay as described above was made on a patient's Sigmoid Colon primary tumor using disassociated tumor cells at 1, 2 and $4x10^5$ cells ml/for the samples. The cell samples were continuously exposed to test drugs in vitro for seven days. Drugs were tested at 200, 100, 50, 25 12.5, 6.25, 3.13 and 1.56 percent of their estimated Peak Plasma Concentration (PPC) in vivo. Tumor growth inhibition was quantitated by ATP luminometry. Interpretations are based on mean percent inhibition at 25, 12.5, 6.25 and 3.13 percent of PPC. The results are tabulated below.

DRUG TESTED	PPC ug/ml	INTERPRETATION
5-FU/LEUCOVORIN	45.0/1.2	PARTIALLY SENSITIVE
5-FU	45.0	PARTIALLY SENSITIVE
LEUCOVORIN	1.2	RESISTANT
MITOMYCIN C	0.9	RESISTANT
ADRIAMYCIN	0.5	RESISTANT
VINBLASTIN	0.8	RESISTANT
CIS-PLATIN	2.5	RESISTANT
MELPHALAN	0.3	RESISTANT
METHOTREXATE	2.8	RESISTANT
BCNU	2.0	RESISTANT

CLAIMS:

1. An ATP release reagent which comprises an aqueous solution of a non-toxic surface active agent in proportions sufficient to render the cell membrane of viable cells permeable to relatively small molecules without disruption of the cell membrane, a buffer for adjusting the pH of a cell suspension to the pH value desired for a bioluminescent test assay of any ATP that becomes released from the cells into the ATP release reagent and Ammonium Vanadate dissolved in the ATP release reagent in amounts effective to stabilize any ATP that becomes dissolved in the ATP release reagent.

2. An ATP release reagent according to claim 1 wherein the non-toxic wetting agent comprises from 0.1 - 0.5% by wt of an ethoxylated phenol wetting agent.

3. An ATP release reagent according to claim 1 wherein the Ammonium Vanadate content therein is about 8 to 14 millimolar.

4. An ATP release reagent according to claim 1 buffered to about pH 7.8.

5. In the method for determining sensitivity of tumor cells to chemotherapeutic agents by:

culturing a predetermined number of disassociated tumor cells suspended in a growth medium in the presence of a chemotherapeutic agent;

extracting ATP from the cultured cells; and

thereafter measuring the quantity of ATP extracted with the firefly bioluminescence reaction, the improvement which comprises:

extracting ATP from the cultured tumor cells by contacting the cells with ATP release reagent comprised of an aqueous

solution of a non-toxic wetting agent in proportions sufficient to render the tumor cell membranes permeable to relatively small molecules without totally disrupting the cell membranes, a buffer for adjusting the pH of the tumor cell suspension to about pH 7.8 and Ammonium Vanadate in ATP stabilizing amounts.

6. A method according to Claim 5 wherein the Ammonium Vanadate content in the ATP extract solution is from 2.6 - 4.6 millimolar.

AMENDED CLAIMS

[received by the International Bureau on 6 October 1992 (06.10.92); original claims 1-6 replaced by amended claims 1-7 (2 pages)]

1. A reagent comprising:

about 0.03 to 0.50 percent by weight non-toxic surface active agent;

about 8 to 14 millimolar ammonium vanadate; and

buffer for adjusting pH to about 7.7 to 7.9, wherein said reagent extracts and stabilizes cellular ATP.

2. The reagent of claim 1 wherein said buffer contains Hepes.

3. The reagent of claim 2 wherein said buffer contains about 0.1 to 0.35 molar Hepes.

4. The reagent of claim 1 wherein said non-toxic surface active agent is Triton-X.

5. The reagent of claim 1 wherein said non-toxic surface active agent is Nonidet P-40.

6. A method for determining sensitivity of tumor cells to chemotherapeutic agents, comprising the steps of:

providing a predetermined number of disassociated tumor cells suspended in a growth medium;

culturing said cells in the presence of a chemotherapeutic agent, said cells being cultured under conditions sufficient for the cells to grow and divide;

extracting and stabilizing ATP from said cells by contacting the cells with a sufficient amount of reagent comprising non-toxic surface active agent, ammonium vanadate, and buffer for adjusting pH to about 7.7 to 7.9; and

measuring said ATP using a bioluminescent reaction.

7. The method of claim 6 wherein said reagent is contacted with the cells in an amount whereby the ammonium vanadate content is from about 2.6 to about 4.6 millimolar.

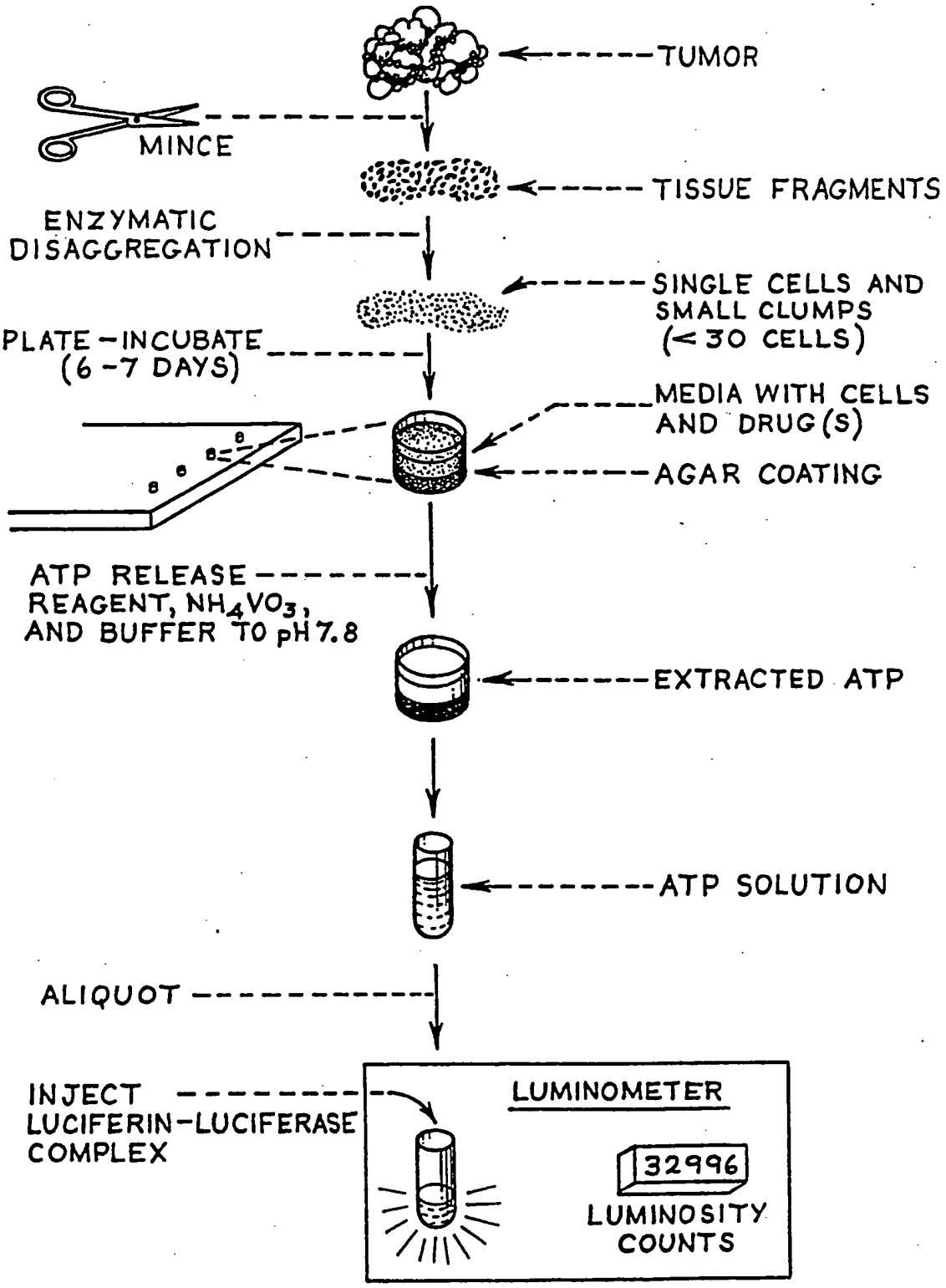

TUMOR
MINCE
TISSUE FRAGMENTS
ENZYMATIC DISAGGREGATION
SINGLE CELLS AND SMALL CLUMPS (< 30 CELLS)
PLATE - INCUBATE (6 - 7 DAYS)
MEDIA WITH CELLS AND DRUG(S)
AGAR COATING
ATP RELEASE REAGENT, NH_4VO_3, AND BUFFER TO pH 7.8
EXTRACTED ATP
ATP SOLUTION
ALIQUOT
INJECT LUCIFERIN-LUCIFERASE COMPLEX
LUMINOMETER
32996
LUMINOSITY COUNTS

Fig. 1

Fig. 2

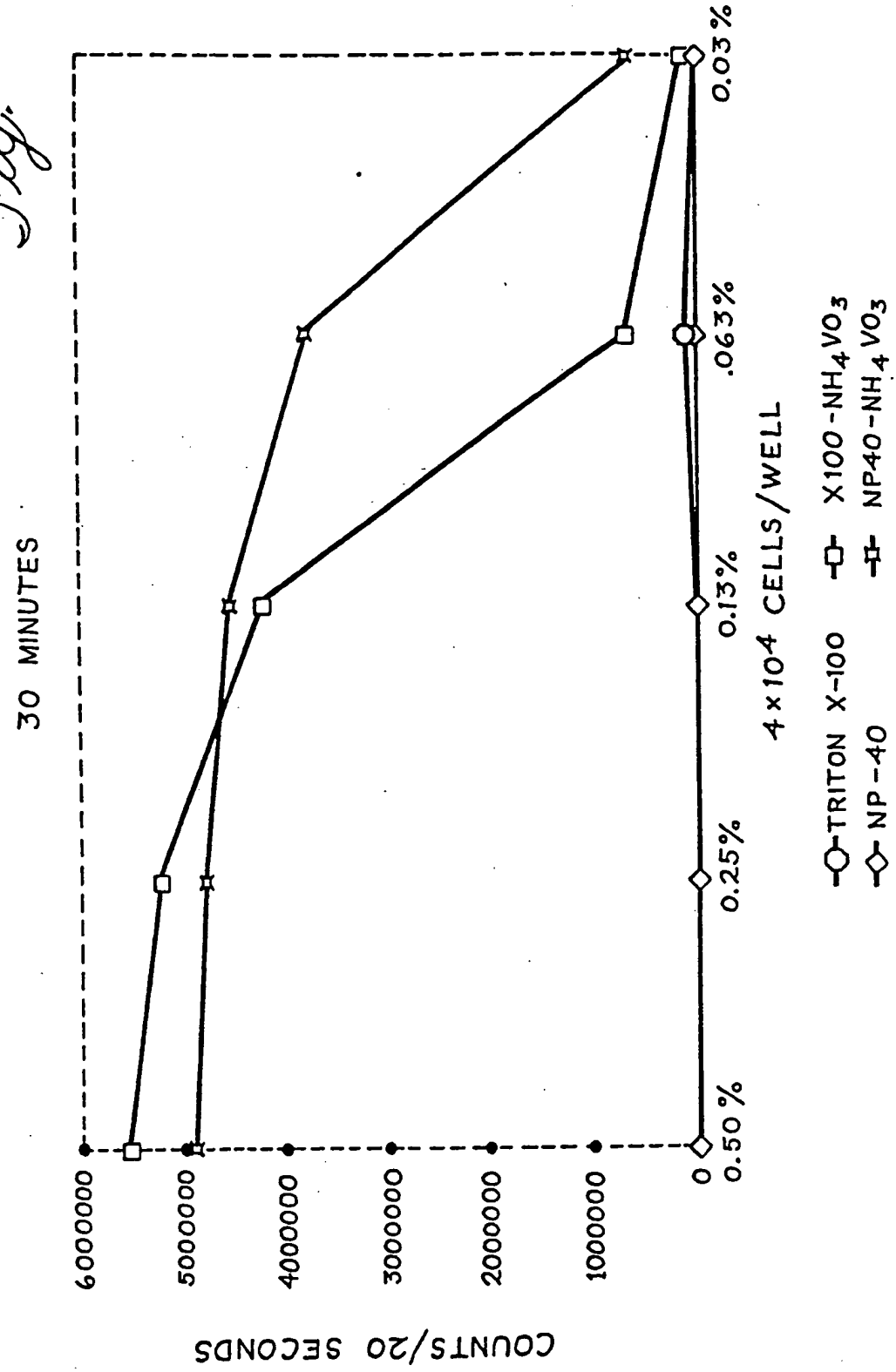
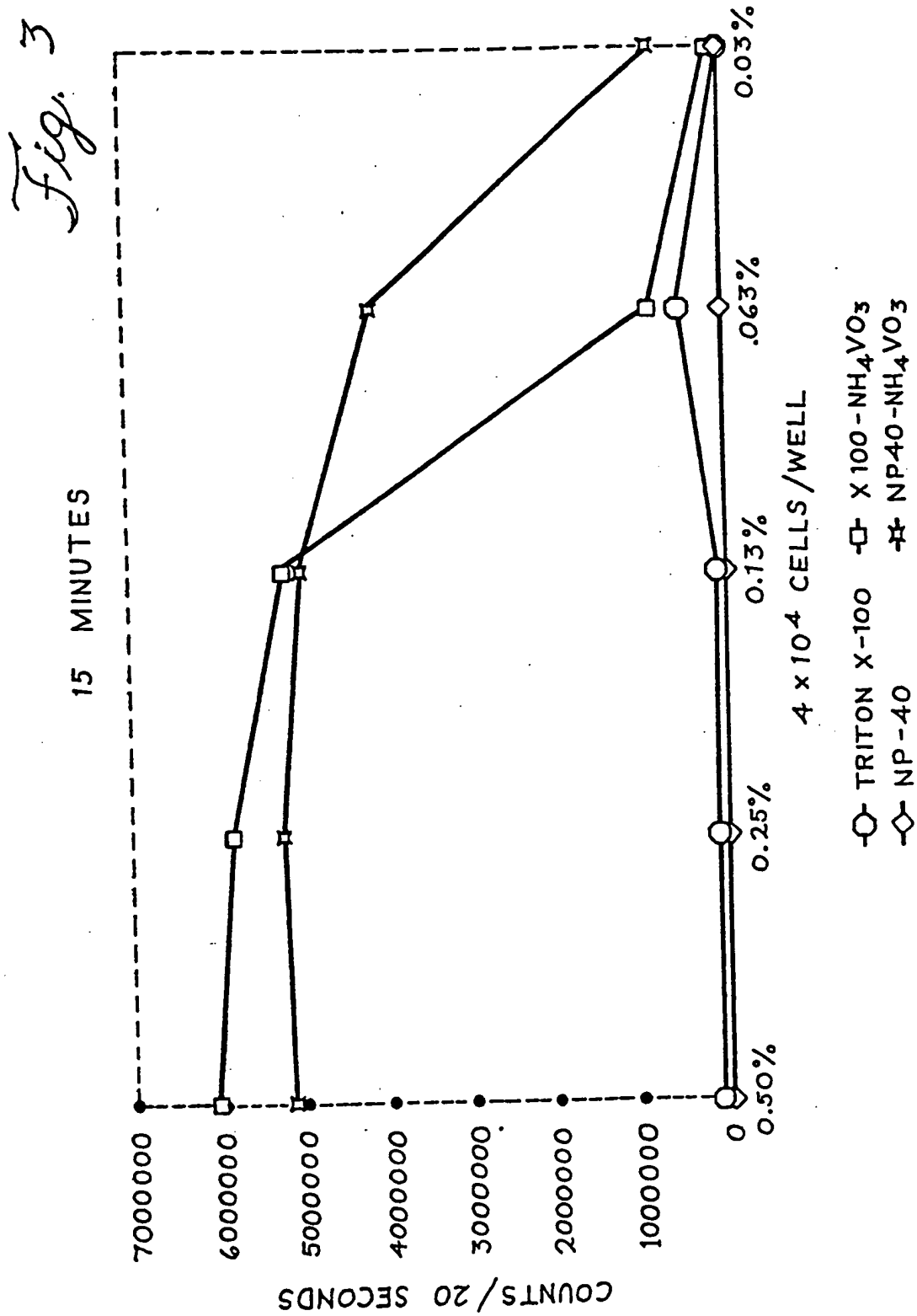

30 MINUTES
COUNTS/20 SECONDS
6000000
5000000
4000000
3000000
2000000
1000000
0
4 x 10⁴ CELLS/WELL
0.03%
.063%
0.13%
0.25%
0.50%
TRITON X-100
NP-40
X100-NH4VO3
NP40-NH4VO3

Fig. 3


15 MINUTES
COUNTS/20 SECONDS
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
0.03%
.063%
0.13%
0.25%
0.50%
4 x 10⁴ CELLS/WELL
TRITON X-100
X 100-NH4VO3
NP-40
NP40-NH4VO3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/04101

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 1/06; C12Q 1/66; C12Q 1/34
US CL : 435/8, 18; 536/27; 428/662

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/8, 18; 536/27; 428/662

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS
seach terms: ammonium (W) vanadate; Andreotti, Peter ?/in

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,303,752 (KOLEHMAINEN ET AL) 01 DECEMBER 1981, see claims 1 - 18.	1 - 6
Y	Journal of Biological Chemistry, Volume 252, No. 21, issued 10 November 1977, Cantley, Jr. et al., "Vanadate Is a Potent (Na,K)-ATPase Inhibitor Found in ATP Derived From Muscle", pages 7421 - 7423, see page 7422, column 2, paragraph 3.	1 - 6
Y	Gynecological Oncology, Volume 31, issued 1988, Sevin et al., "Application of an ATP-Bioluminescence Assay in Human Tissue Chemo-sensitivity Testing", pages 191 - 204, see entire document.	5 - 6

* Special categories of cited documents:[15]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
30 JUNE 1992	10 AUG 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	GARY L. KUNZ

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Optimization of the Firefly Luciferase Assay for ATP[1]

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WEBSTER, J. J., AND LEACH, F. R. Optimization of the Firefly Luciferase Assay for ATP. *J. Appl. Biochem.* 2, 469–479 (1980).

The conditions for maximum light emission in ATP analyses using firefly lantern extracts, partially purified luciferase preparations, and crystalline luciferase were determined. Tricine buffer (pH 7.8) used at a final concentration of 0.025 M was found to be the best of several buffers tested. Mg^{2+} was required and its optimum concentration was 5 mM. The relative amounts of luciferase and luciferin influenced light output; for maximum light production the luciferase preparations should be supplemented with extra luciferin. Under normal assay conditions oxygen was not limiting. Bovine serum albumin, EDTA, and dithiothreitol were all found to stabilize luciferase and yield more reproducible results. Modification of the sample holder for the SAI Model 3000 photometer and the use of 0.2 ml reaction volume increased the measured light output. ATP standard graphs constructed by using chart recorder peak height, instrument peak height, and integration mode determination of light output consisted of parallel lines. The conditions for reagent stability were defined.

INTRODUCTION

The availability of commercial firefly luciferase reagents and of instrumentation for measuring light production is markedly increasing the analytical application of bioluminescence. Three symposia have been devoted to determination of ATP and the analytical application of bioluminescence and chemiluminescence (1–3). Several methods of luminescent analysis were described in Methods in Enzymology, Vol. 57 (4), and a recent monograph (5) has provided a comprehensive treatment of bioluminescence. Webster *et al.* (6) have compared several characteristics of the commercially available firefly luciferase preparations. In this paper we report the results of experiments aimed at optimization of the firefly luciferase assay of ATP using commercially available equipment and reagents.

MATERIALS AND METHODS

Enzymes and chemicals. Luciferase preparations were obtained from E. I. Du Pont de Nemours and Company and from the Sigma Chemical Company. The luciferase from Du Pont (120 mg dry wt) was dissolved in 3 ml of the appropriate buffer (0.05 M, pH 7.8) containing 10 mM $MgSO_4$, 1 mM EDTA, and 1 mM dithiothreitol. Sigma firefly lantern extract FLE-50 was reconstituted in 5 ml of water to give a solution (pH 7.4) containing 0.05 M potassium arsenate and 0.02 M $MgSO_4$. Sigma Type IV luciferase (crystalline, 1 mg) was dissolved in 200 μl of 10%

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TABLE I

Effect of Various Buffers on Luciferase Activity[a]

Buffer[b]	pK_a (20°C)	Activity relative to that in Hepes
Mops	7.20	0.65
Phosphate	7.21	0.09
Tes	7.50	0.54
Hepes	7.55	1.00
Hepps	8.00	0.68
Tricine	8.15	1.25
Glycinamide	8.20	0.80
Tris	8.30	1.00
Bicine	8.30	0.70
Glycylglycine	8.40	0.72

[a] The activity of Du Pont luciferase (400 μg dry wt) was determined in solutions of the indicated buffers of pH 7.8. Reaction mixtures (1 ml) contained 50 ng ATP, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT.

[b] Concentration of each, 0.025 M.

ammonium sulfate and then diluted to 1 ml to give a solution containing 0.05 M Tricine,[2] 10 mM $MgSO_4$, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mg of bovine serum albumin. Luciferin was synthesized by Dr. A. S. Radhakrishna in the laboratory of Dr. K. D. Berlin. Arsenite was obtained from Mallinckrodt, Chelex from Bio-Rad, DTT from Calbiochem, and EDTA from Fisher. Other enzymes and chemicals were purchased from the Sigma Chemical Company.

Light measurement. Light production was measured in a SAI Technology Model 3000 ATP photometer equipped with a Houston Instrument Omni Scribe Model 35217-5 recorder. Using a luminol standard, 1.4×10^4 photons produced the voltage recorded as one count on the digital readout. Thus 1000 counts (1.4×10^7 photons) represents the production of one light unit.

RESULTS AND DISCUSSION

Assay Components

Buffer. Several characteristics of the luciferase reaction mixture influence luciferase activity. Thus, there is an anion-binding site on luciferase which influences enzymatic activity and the wavelength of light emitted from the activated luciferin depends on pH (8). Webster *et al.* (9) have found that firefly luciferase has different conformations that give differences in enzymatic activity depending upon the buffer used. Formerly, arsenate buffer was used by Strehler and Totter (10) to inhibit luciferase and thus slow down the light production so that measurements could be made with the instruments available at that time. McElroy (11) has recently reminded users of firefly luciferase that arsenate is no longer required with

[2] Abbreviations used: Mops, 3-(*N*-morpholino)propanesulfonic acid; Tricine, N-[tris(hydroxymethyl)methyl]glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tes, 2-[tris(hydroxymethyl)methylamino]ethanesulfonic acid; Hepps, 4-(2-hydroxyethyl)piperazine-propanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; DTT, dithiothreitol; BAL, British anti-Lewisite, 2,3-dimercapto-1-propanol.

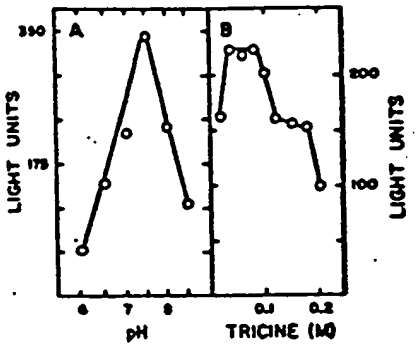

FIG. 1. pH optimum and effect of buffer concentration on luciferase activity. (A) Tricine buffer (0.025 M) at various pH values used with 100 ng ATP, 400 μg Du Pont luciferase-luciferin, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT. (B) Various Tricine buffer concentrations of pH 7.8 used. The reaction system (1 ml) contained 100 ng ATP, and 0.1 mM Mg^{2+}.

the current light detectors and fast recorders now available, and can only lower the sensitivity of the assay.

Table I lists several buffers with pK_a values between 7.20 and 8.40 and shows the activity of luciferase in each buffer. Tricine-buffered reaction mixtures yielded the greatest light production. There appears to be no relationship between the pK_a of the buffer and the luciferase activity.

Because of the low enzymatic activity observed in phosphate buffer (possibly due to the presence of heavy metals (11)) both the phosphate and Tricine buffers were treated with Chelex (12). Since there was only a slight increase in enzymatic activity, metal contamination was not considered significant.

The optimum pH for luciferase activity was 7.8 (see Fig. 1A). This optimum is consistent with that observed by Green and McElroy (13). The effect of various buffer concentrations on enzymatic activity is shown in Fig. 1B. With buffer concentrations greater than 0.1 M there was considerable inhibition of luciferase activity. Since firefly luciferase is an euglobulin some salt is required to keep it in solution. Tricine buffer (0.025 M) of pH 7.8 was used in the standard ATP assay system.

Magnesium ion concentration. Mg^{2+} reacts with ATP to form a magnesium ion–ATP complex which is the actual substrate for luciferase (4). The following Mg^{2+} salts were equally suitable: $MgSO_4$, $MgCl_2$, and $Mg(O_2CCH_3)_2$. Figure 2 shows the effect of different Mg^{2+} concentrations on enzymatic activity—a final Mg^{2+} concentration of 5 mM was used.

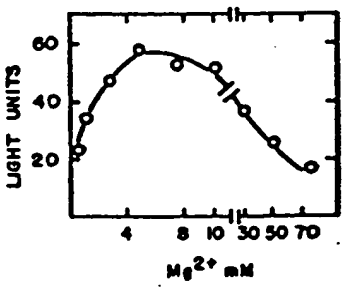

FIG. 2. Effect of Mg^{2+} concentration on luciferase activity. The reaction system (1 ml) contained 400 μg Du Pont luciferase-luciferin, 10 ng ATP, 0.5 mM DTT, and various amounts of Mg^{2+}.

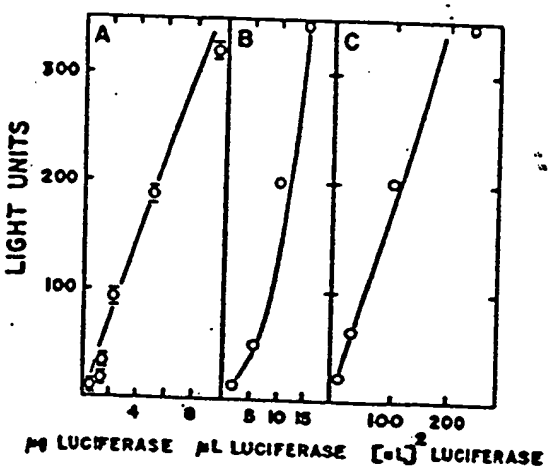

FIG. 3. Effect of luciferase concentration on light production. (A) Various concentrations of Sigma Type IV used in the 1-ml reaction system containing 1 ng ATP, 50 μg luciferin, 0.025 M Tricine, pH 7.8, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT. (B) Various concentrations of the Du Pont luciferase–luciferin preparation were used in a reaction mixture (1 ml) containing 5 ng ATP, 0.025 M Tricine, pH 7.8, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT. (C) Data from (B) replotted using as abscissa the square of the volume of luciferase–luciferin preparation used.

Luciferase. Figure 3 shows the light output obtained using various amounts of Sigma Type IV luciferase (A) and Du Pont luciferase (B). Figure 3A shows that the light produced by Sigma Type IV enzyme is proportional to protein concentration. However, the addition of increasing volumes of Du Pont luciferase–luciferin reagent (Fig. 3B) does not result in a linear increase in light production because the amount of luciferin present in the preparation is limiting. Supplementation of the Du Pont luciferase–luciferin-containing reaction mixture with 50 μg of luciferin produced a linear response (data not shown). Figure 3C shows a plot of enzymatic activity against the square of the volume of the Du Pont luciferase–luciferin reagent to be linear. Dilution of the mixed luciferase–luciferin reagents has a marked effect on light production unless the diluted reagent is supplemented with luciferin. The light production from a given quantity of ATP varies depending upon the relative luciferase and luciferin concentrations (6).

Luciferin. The effect of adding various concentrations of luciferin to a luciferase preparation that is completely luciferin-dependent is shown in Fig. 4. Most of the commercial reagents that contain luciferin are not saturated with luciferin (6). Karl and Holm-Hansen (14) have shown that additional luciferin increased the response

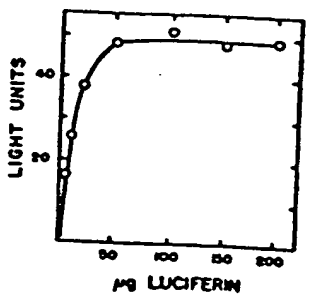

FIG. 4. Effect of luciferin concentration on light production. The reaction system (1 ml) contained 0.025 M Tricine buffer, pH 7.8, 2 μg Sigma Type IV luciferase, 1 ng ATP, 5 mM Mg^{2+}, 0.5 mM EDTA, 0.5 mM DTT, and various amounts of luciferin.

TABLE II

Effects of Additions to or Omissions from the Reaction System on Light Production by Firefly Luciferase[a]

	Activity (light units)		
	Sigma FLE-50	*Du Pont*	*Sigma Type IV*
Part A. Additions			
None	1.1	1.3	0.2
Mg^{2+}	1.6	1.7	0.1
EDTA	0.1	0.2	0.1
Luciferin	70.4	9.5	2.0
DTT	1.2	1.5	0.1
Bovine serum albumin	2.4	1.7	0
Part B. Omissions			
None	37.9	12.0	4.3
Mg^{2+}	19.9	1.1	0
EDTA	32.0	11.1	4.4
Luciferin	0.5	1.9	0
DTT	25.0	10.5	4.0
Bovine serum albumin	19.4	11.3	2.0

[a] Reactions were conducted in Bio-Vials using 100 pg of ATP, and 0.025 M Tricine buffer, pH 7.8, with peak height measurements of light emission. The amounts of luciferase used were: Du Pont, 400 μg; Sigma FLE-50, 400 μg; Sigma Type IV, 2 μg. The other reaction components were either added individually (Part A) or omitted individually (Part B) from a reaction mixture containing all of the basic components. The concentrations used in the final reaction mixture (1.0 ml) were: luciferin, 50 μg; bovine serum albumin, 100 μg; Mg^{2+}, 5 mM; EDTA, 0.5 mM; and DTT, 0.5 mM.

given by Sigma FLE-50 lantern extracts. They found that aging of the enzyme preparations was required to reduce the background. For greatest sensitivity in measuring ATP most commercial preparations require added luciferin.

Oxygen. Oxygen is a substrate in the luciferase-catalyzed reaction. Under normal assay conditions no stimulation was observed by bubbling oxygen through the Tricine buffer solution for up to 30 min before use. To demonstrate an oxygen requirement glucose and glucose oxidase were incubated with the luciferase reaction mixture prior to addition of ATP. Either of those two reagents alone reduced the light output only 7–9% while in combination they reduced light output by 77%. Under normal assay conditions oxygen was not limiting.

Effects of Other Additions to and Omissions from the Assay System. Three typical luciferase preparations were compared because they represented the three levels of purity commercially available: Sigma FLE-50, a crude lantern extract; Du Pont, a partially purified luciferase reagent; and Sigma Type IV, a crystalline luciferase. Table II, part A, shows the effect of various additives. The activity of the FLE-50 preparation was stimulated by Mg^{2+}, luciferin, and bovine serum albumin. Light production by the Du Pont luciferase was stimulated by luciferin. Single additions to the Sigma Type IV luciferase resulted in only slight increases because most of the required components were not present in the assay mixture. In each case EDTA by itself was inhibitory. The effects of omissions from reaction mixtures containing all of these components are shown in Table II, part B. All luciferase preparations had decreased activity without additional luciferin.

TABLE III

Reversal of Arsenate Inhibition of Firefly Luciferase[a]

Substance added	Light units	Percentage of control
Experiment A		
None	8.2	49
0.1 M Phosphate	2.7	61
400 μg Luciferase–luciferin	30.2	180
10 mM $MgSO_4$	7.5	45
10 ng Pyrophosphate	6.3	38
5 ng AMP	7.4	44
Experiment B		
None	34.4	46
2 μg Luciferase	91.9	123
50 μg Luciferin	37.3	50

[a] Reaction mixtures (1 ml) contained 0.025 M Tricine buffer, pH 7.8, 5 ng ATP, 0.01 M arsenate, 5 mM $MgSO_4$, 0.5 mM EDTA, and 0.5 mM DTT. Integrated counts were measured for a 60-s period after a 15-s delay. For Experiment A 400 μg of Du Pont luciferase–luciferin mixture was used and the arsenate-free mixture produced 16.8 light units, which was used as the control value. In Experiment B, 2 μg of Sigma Type IV luciferase and 50 μg of luciferin were used. The arsenate-free reaction produced 74.6 light units, which was used as the control value.

Because of the essential nature of the sulfhydryl groups (15) DTT is included in the complete reaction mixture. Bovine serum albumin was stimulatory with both Sigma preparations (reduced light output when omitted). Many of the partially purified luciferase preparations are supplemented with bovine serum albumin to increase stability. Mg^{2+} is required by all preparations.

Inhibitors

Ionic strength effects. Denburg and McElroy (8) showed that luciferase has one anion-binding site per active site and that all anions bind at the same site. The ionic strength effect increases the K_m for the magnesium ion–ATP complex. The effect

TABLE IV

Inhibition of Luciferase by Arsenite and 2,3-Dimercapto-1-propanol[a]

Buffer	Arsenite concentration (M)	Activity (light units)[b]	
		No addition	0.01 M BAL
Phosphate buffer, 0.025 M	0	118.5 [100]	38.9 [33]
	0.01	142.3 [120]	9.7 [8]
	0.1	68.3 [58]	5.1 [4]
Tricine buffer, 0.025 M	0	109.2 100]	56.1 [51]
	0.01	87.2 [80]	25.9 [24]
	0.1	4.6 [4]	0 [0]

[a] Reaction mixtures (1 ml) contained the indicated buffer, 5 mM $MgSO_4$, 0.5 mM EDTA, 400 μg of Du Pont luciferase, and 50 ng of ATP.

[b] Values in brackets show the activity as percentage of control without added arsenite or BAL.

TABLE V

Effect of Volume and Cuvette Size on Measured Light Production[a]

Reaction volume (ml)	ATP (pg)	Luciferase (μg)	Light units		
			Scintillation vial	Beckman Bio-Vial	6 × 50-mm tube
1	1000	400	33.7	47.8	
0.5	500	200		27.6	14.3
0.5	100	200	3.0	5.8	1.8
0.2	100	200		26.7	16.8

[a] Du Pont luciferase–luciferin was used in the amounts indicated. The reaction system contained 0.025 M Tricine buffer, pH 7.8, 5 mM $MgSO_4$, 0.5 mM EDTA, and 0.5 mM DTT.

of arsenate on luciferase is not specific (18). Table III shows which component of the reaction mixture reverses the inhibition of light production by arsenate. Part A shows that an increase in the concentration of luciferase–luciferin reversed the inhibition. When pure luciferase and luciferin were used (Table III, part B), the luciferase component was active in reversing arsenate inhibition. This reversal would operate by increasing the number of binding sites for the anions.

Sulfhydryl reagents. DeLuca *et al.* (15) have shown that there are two essential sulfhydryl groups in luciferase. We found that dithiothreitol gave a 1.6-fold stimulation of light production while both mercaptoethanol and cysteine gave 1.1-fold stimulations. Dithiothreitol was effective in reversing *p*-chloromercuribenzoate inhibition. The existence of two essential sulfhydryl groups raises the question of their proximity; this was examined using arsenite. British anti-Lewisite is known to reverse arsenite inhibition specifically. When BAL was used in an attempt to reverse arsenite inhibition of luciferase, BAL itself was seen to be inhibitory and arsenite and BAL each potentiated the inhibition produced by the other compound (Table IV). Results are shown using both phosphate buffer and Tricine buffer for the assay of luciferase. We have shown previously that luciferase has different conformations depending upon which buffer is used (9).

REACTION AND MEASUREMENT CONDITIONS

Assay Volume

Table V shows results obtained using reaction vessels of different sizes and different reaction volumes in the SAI Model 3000 photometer. With a 1-ml reaction volume, change from a standard 25-mm-diameter glass scintillation vial to a 10-mm-diameter plastic vial increased the measured light output by 40%. A further reduction in the diameter of the reaction vessel to 6 mm, rather than further increasing the measured light output, decreased it by about 40%. A reduction of reaction volume from 0.5 to 0.2 ml in the Bio-Vial gave about a fivefold increase in the measured light output.

Temperature

The optimum temperature for light output was 25°C, as reported by McElroy and Strehler (17). At 30°C there was a 20% reduction in light output while at 20°C there was only a 5% reduction in light output.

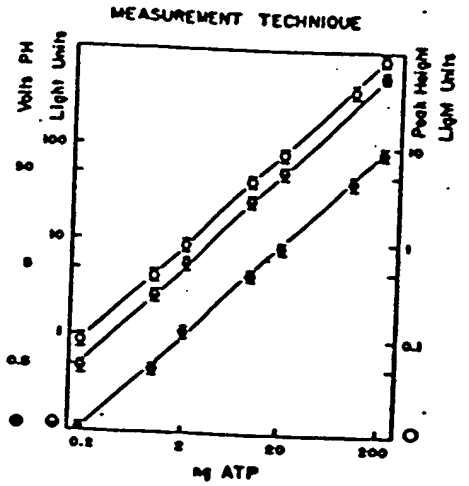

FIG. 5. Measurement of luciferase activity by peak height voltage, peak height counts, and integration of light production. The light output from reaction mixtures (1 ml) containing 0.025 M Tricine buffer, pH 7.8, 5 mM Mg^{2+}, 0.5 mM EDTA, 0.5 mM DTT, 400 μg of Du Pont luciferase–luciferin was determined.

Measurement Conditions

DeLuca *et al.* (18) suggested that integrating total light output for an arbitrary time could lead to serious errors in the determination of ATP content at different final concentrations of ATP. However, they also stated that no single method of measuring light production was adequate for all conditions used for ATP analysis. Therefore, we compared three methods for measuring light production using the SAI Model 3000 photometer. Figure 5 shows parallelism between the dependence of light output on ATP concentration when light output was measured by the peak height determined by recorder trace, by the counts when the photometer was used in the peak height mode (a 2-s delay followed by a 1-s count), and by counts determined in the integration mode (15-s delay, 60-s count).

STABILITY OF REAGENTS

For routine assays it is convenient to prepare stock solutions that can be stored. Experiments were done to define suitable conditions for storage and the length of time that the reagents remained useful.

Luciferase

The stability of luciferase in solution was measured for up to 50 days of storage (Table VI). The enzyme was completely stable in either phosphate or Tricine buffer for at least 24 h. There was little activity loss until 10 days after preparation. The enzyme preparations were useful for up to 3–4 weeks if ATP standards were used to correct for the loss of activity after 10 days. A sufficient amount of activity remained after 50 days for the detection of 1 ng of ATP.

Another way to store the luciferase was to freeze the preparation. Table VII shows that luciferase lost about 15% of its activity when thawed and refrozen on 4 consecutive days. After 4 weeks of storage in frozen state there was only a 15–25% loss and after 8 weeks of storage in this way there was 9–37% loss.

Spiegel and Tifft (18) observed 90% loss of luciferase activity when Du Pont

TABLE VI

Stability of Luciferase on Storage[a]

Time	Residual activity (%)	
	Tricine buffer	*Phosphate buffer*
1 hr	95	86
3 hr	90	96
5 hr	106	101
24 hr	102	111
2 days	104	115
4 days	109	109
7 days	94	115
10 days	96	120
14 days	75	104
21 days	57	86
28 days	63	—
49 days	22	65

[a] Du Pont luciferase–luciferin in Tricine or phosphate buffer was stored for the indicated time at 4°C. The enzymatic activity was determined in a 1-ml reaction system containing 10 ng ATP, 0.025 M Tricine, pH 7.8, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT.

luciferase–luciferin reagent preparations were stored refrigerated in the dark for 4 days. The preparation was stable for 4 days if stored frozen and in the dark.

ATP

ATP solutions of concentration 5 ng/ml were prepared in Tris, phosphate, Mops, and Hepes buffers, and the light output each produced was measured at various

TABLE VII

Stability of Luciferase on Freezing and Thawing[a]

Time (days)	Residual activity (%)	
	Du Pont	*Sigma Type IV*
Repeated freeze-thaw treatment		
2	80	81
3	75	78
4	80	92
Single freeze-thaw treatment, different lengths of storage		
7	73	75
14	75	81
28	74	85
56	63	91

[a] The Du Pont luciferase–luciferin and the Sigma Type IV luciferase were stored at −15°C. The same sample was frozen and thawed each day for the results in the upper part of the table. The results in the lower part of the table were obtained with individual samples thawed for test after the appropriate storage period. The reaction system (1.0 ml) contained 5 ng ATP, 0.025 M Tricine, pH 7.8, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT. With the Sigma Type IV preparation luciferin/50 μg was added.

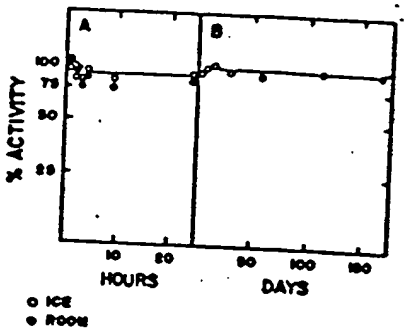

FIG. 6. Stability of luciferin upon storage. Luciferin (5 mg/ml) was dissolved in sterile water and stored in Beckman Bio-Vials wrapped with aluminum foil and flushed with nitrogen. The reaction system (1 ml) contained 0.025 M Tricine, pH 7.8, 1 ng ATP, 5 mM Mg^{2+}, 0.5 mM EDTA, 0.5 mM DTT, and 400 μg of Du Pont luciferase–luciferin. A 12-fold increase in light output is produced by the additional 50 μg of luciferin. For (A) the luciferin was stored on ice (4°C) and at room temperature (20°C). For (B) the luciferin was stored frozen.

times during a 26-h storage period at room temperature (22–25°C). The solutions in Tris, Mops, and Hepes buffers were completely stable for 6 h after which time a slight drop occurred. The stability in phosphate buffer was less. Stock solutions of ATP of concentration 10 μg/ml or greater can be stored in deionized water or Tris buffer without serious loss for at least a year if kept frozen and sterile. Spiegel and Tifft (18) found no problems with the stability of ATP standards during a 4-day period.

Luciferin

When luciferin preparations (concentration 1 mg/ml) are stored either on ice or at room temperature (21°C) and stored under N_2 in a tightly capped vial, there is little loss of luciferin (Fig. 6A). Figure 6B shows that luciferin at 5 mg/ml is stable for 24 weeks when stored frozen (−15°C) under nitrogen.

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Effect of Solvents on the Catalytic Activity of Firefly Luciferase[1,2]

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Various solvents stimulate the catalytic activity of firefly luciferase, up to sevenfold. Polyvinylpyrrolidone, polyethylene glycols, and nonionic detergents such as Triton X-100 were the most effective stimulators of the enzyme. Both peak light and total light emission were enhanced in the presence of these solvents indicating an increased turnover of the enzyme. The primary effect of the solvents is on the oxidative reaction rather than the activation reaction. All the experimental data support the hypothesis that the presence of solvent promotes the dissociation of the inhibitory product from the enzyme.

Firefly luciferase from *Photinus pyralis* has been extensively studied by many investigators since it was first crystallized by Green and McElroy in 1956 (1). The enzyme catalyzes the oxidative decarboxylation of luciferyl-adenylate as shown in the following reactions:

$$\text{D-LH}_2^{5} + \text{ATP} + \text{E} \overset{Mg^{2+}}{\rightleftharpoons} \text{E}\cdot\text{LH}_2\text{AMP} + PP_i \quad [1]$$

$$\text{E}\cdot\text{LH}_2\text{AMP} + \text{O}_2 \rightarrow \text{E}\cdot\text{P} + \text{CO}_2 + \text{AMP} + h\nu \quad [2]$$

The first reaction [1] is the activation of luciferin to form an enzyme bound luciferyl adenylate and PP_i. This reaction is relatively specific for ATP and in order for reaction [2] to proceed D-LH_2 is required, L-LH_2 is inactive for light emission. Reaction [2] is the oxidative decarboxylation of the bound luciferyl adenylate and produces an enzyme-product complex, CO_2, AMP, and light. When substrates are present at saturating concentrations, the enzyme is strongly inhibited by the product, oxyluciferin, and normally turns over only twice (2). In addition to the above reactions, luciferase will also catalyze reaction [3] the activation of the competitive inhibitor dehydroluciferin (L) to form a tightly bound complex, E·LAMP.

$$\text{L} + \text{ATP} + \text{E} \overset{Mg^{2+}}{\rightleftharpoons} \text{E}\cdot\text{L-AMP} + PP_i \quad [3]$$

Since dehydroluciferin cannot be oxidized the reaction stops at E·L-AMP.

High concentrations of AMP, PP_i, and anions such as SO_4^{2-} are inhibitors of the enzyme (3). Prior to this study there were no examples of substances that would

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[5] Abbreviations used: E, firefly luciferase enzyme; L, dehydroluciferin; LH_2, luciferin; Tricine, *N*-tris(hydroxymethyl)-methyl glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, polyethylene glycol; PVP, polyvinylpyrrolidone; BSA, bovine serum albumin; CMC, critical micelle concentration.

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stimulate enzymatic activity. In this paper we present results of the effects of various solvents on the catalytic activity of luciferase. Enzymatic activity was stimulated by these solvents up to sevenfold.

MATERIALS AND METHODS

Glycylglycine, Tricine, and Hepes were obtained from Sigma Chemical Company, St. Louis, Missouri 63178. Adenosine 5'-triphosphate and bovine serum albumin (A grade) were obtained from Calbiochem-Behring Corporation (La Jolla, Calif. 92122). Luciferin and dehydroluciferin were synthesized as described previously (4). Firefly luciferase was purified according to the procedure of Green and McElroy (1). Bacterial luciferase was purified from a frozen cell paste of *Beneckea harveyi*, and was assayed as described previously (5). Inorganic [^{32}P]pyrophosphate, specific activity 20 mC/μmol, was purchased from New England Nuclear, Boston, Massachusetts 02118.

Solvents

Nonionic. Polyethylene glycol (PEG, approximate molecular weights 600 and 8000), polyvinylpyrrolidone (PVP, average molecular weight 40,000), dextran (clinical grade, average molecular weight 173,000), and polyoxyethylene sorbitan monolaurate (Tween 20), were purchased from Sigma Chemical Company. PEG (Aquacide III; average molecular weight 20,000) was obtained from Calbiochem-Behring Corporation. Ethylene glycol, propane-1,2-diol, and glycerol were supplied by Mallinckrodt Inc., Paris, Kentucky 40361. Triton X-100 (average molecular weight 646) was obtained from A. J. Lynch and Company, Los Angeles, California 90058. Lauryl maltoside and octyl glucoside were gifts from Professor Shelagh Ferguson-Miller (Department of Biochemistry, Michigan State University, East Lansing, Mich. 48823).

A sample of pure PEG 8000 (without added antioxidants) was kindly supplied by Dr. Louis F. Theiling, Union Carbide Corporation, South Charleston, West Virginia.

Cationic. Cetyltrimethyl ammonium bromide was purchased from Sigma Chemical Company.

Anionic. Sodium lauryl sulfate and sodium deoxycholate were obtained from Sigma Chemical Company.

Zwitterionic. Zwittergent 3-14 (*N*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate) was purchased from Calbiochem-Behring Corporation.

Firefly luciferase assay. The stock assay mixture consisted of 8 ml glycylglycine buffer (0.025 M, pH 7.8), 0.5 ml magnesium sulfate (0.1 M, pH 7.8), and 0.8 ml luciferin (10^{-3} M). Luciferase (10 μl, 80 pmol/ml) was added to 400 μl of assay mixture, and the reaction was initiated by injection of 100 μl of 0.02 M ATP. The peak light intensity was recorded with an Aminco Chem-Glow photometer (American Instruments Company, Silver Springs, Md. 20910). Total light emitted was calculated from the area under the light-time curve.

Investigation of Effect of Different Solvents on the Bioluminescent Firefly Luciferase Reaction

Solvents were prepared in phosphate buffer (0.1 M, pH 7.0). The pH of the solutions was adjusted to pH 7.0 using either sodium hydroxide or hydrochloric acid.

The solvent (50 μl) or as a control, 0.1 M phosphate buffer, pH 7.0 (50 μl), was added directly to the assay mixture (350 μl). The enzyme was then added to this mixture and luciferase activity determined as described previously.

In order to achieve very high solvent concentrations in the assay mixture, the solvents were made up to the appropriate concentration in the glycylglycine buffer and used instead of this buffer in the assay mixture. A range of concentrations of the following solvents was studied: PEG 600 (0-50 g/liter), PEG 8000 (0-50 g/liter) PEG 20,000 (0-25 g/liter), PVP (0-20 g/liter) dextran (0-10 g/liter) Triton X-100 (0-16 mM), Tween 20 (0-8.2 mM), ethylene glycol (0-0.8 M), propane-1,2-diol (0-0.66 M), glycerol (0-0.54 M), sodium deoxycholate (0-1 mM), sodium lauryl sulfate (0-3.5 mM), cetyl trimethyl ammonium bromide (0-6.9 mM), lauryl maltoside (0-10 mM), octyl glucoside (0-20 mM), and Zwittergent 3-14 (0-1 mM).

Spectra of emitted light. The spectrum of the light emitted by firefly luciferase reaction in the presence of PVP, PEG, and Triton was measured using a Spectrofluorometer Mark I (Farrand Optical Co., Inc., NY). The reaction was initiated by adding ATP and allowed to proceed for 30 s until the light output was constant; then the emission spectrum was measured.

^{32}P-Inorganic pyrophosphate exchange. $^{32}PP_i$ exchange into ATP was measured as a function of time in the presence of 1×10^{-4} M dehydroluciferin and 5 μg of enzyme. The assay conditions were the same as described by Mehler and Stern (6).

L-AMP titration. The number of binding sites for L-AMP on the enzyme can be determined by measuring the fluorescence of dehydroluciferin in the presence of enzyme and ATP-Mg^{2+}. The enzyme bound L-AMP is essentially nonfluorescent whereas free L is fluorescent (7).

Aliquots of L are added to the enzyme and ATP-Mg^{2+} and the fluorescence is monitored after each addition. When all of the sites are saturated with L-AMP, further addition of L results in a marked increase in fluorescence. In a typical experiment 4×10^{-6} M luciferase in 0.025 M glycylglycine, pH 7.8,

containing 2×10^{-3} M ATP and 5×10^{-3} M $MgSO_4$ was mixed. L was added in 10-μl increments and the fluorescence measured after each addition. Each addition increased the concentration of L in the cuvette by 5×10^{-7} M. Fluorescence was measured on a Perkin-Elmer MPF-44A Spectrofluorometer with an exciting wavelength of 358 nm and emission at 551 nm.

Effect of protein on solvent stimulation of the firefly luciferase reaction. Luciferase assay buffer (350 μl), 50 μl of PEG 8000 (250 g/liter), 5, 10, 25, 50, or 100 μl of BSA (80 mg/ml) in phosphate buffer (0.015 M, pH 7.4) containing sodium chloride (3 g/liter), and 10 μl of firefly luciferase in phosphate buffer (0.1 M, pH 7.0) were placed in an assay tube. The total volume was adjusted to 500 μl by addition of phosphate buffer (0.1 M, pH 7.0). A 100-μl aliquot of ATP (0.02 M, pH 7.0) was then injected and the peak light emission measured. Similar experiments were performed with 10 and 40 mg/ml BSA solutions. The effect of BSA on the PVP-, Triton-, lauryl maltoside-, and Zwittergent-stimulated firefly reaction was investigated in a similar fashion.

Polyacrylamide disc gel electrophoresis. This was carried out in 7.5% gels in 0.05 M phosphate buffer, pH 8.0, for 4 h at 80 V. Samples of luciferase (10 μg) and as a control catalase (10 μg) were electrophoresed in the presence and in the absence of Triton (2 mM), PEG 20,000 (25 g/liter) and PVP (5 g/liter).

RESULTS

Table I summarizes the data obtained with various solvents. Stimulation or inhibition of firefly luciferase activity was dependent on the concentration of the solvent in all cases.

Figure 1 illustrates the concentration dependence of the stimulation by various

TABLE I

EFFECTS OF SOLVENTS ON THE CATALYTIC ACTIVITY OF FIREFLY LUCIFERASE

	Optimal final concentration	Relative activity based on peak light emission (% control)
Solvent		
A. Nonionic		
Ethylene glycol	0.8 M	200
Propane-1,2-diol	0.66 M	200
Glycerol	0.54 M	293
PEG 600	50 g/liter (83 mM)[a]	320
PEG 8000	50 g/liter (6.25 mM)[a]	327
PEG 20,000	25 g/liter (1.15 mM)[a]	382
PVP 40,000	5 g/liter (0.115 mM)[a]	640
Dextran 173,000	10 g/liter (58 μM)	136
Micelle-forming solvents		
B. Nonionic		
Triton X-100	0.75 mM	500
Tween 20	0.82 mM	450
Lauryl maltoside	0.5 mM	545
Octyl glucoside	9 mM	200
C. Cationic		
Cetyltrimethyl ammonium bromide	6.9 mM	−100[b]
D. Anionic		
Lauryl sulfate	3.5 mM	−100
Deoxycholate	1 mM	−100
E. Zwitterionic		
Zwittergent 3-14	1 mM	544

[a] Molar concentration based on the average molecular weight.
[b] Concentration at which complete inhibition was observed.

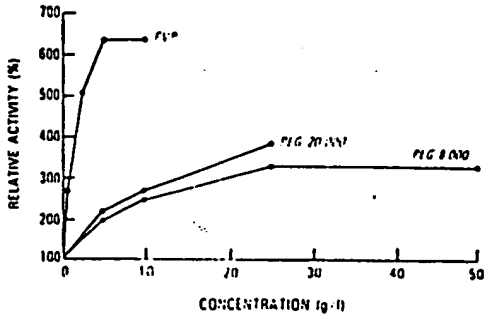

FIG. 1. Concentration dependence of stimulation of peak light emission of a firefly luciferase reaction by PVP, PEG 8000, and PEG 20,000. Concentration shown is final concentration in assay tube.

nonionic substances (Group A, Table I). Stimulation was observed in a variety of buffers (glycylglycine, phosphate, Hepes, Tricine). PEG stimulation increased with increasing average molecular weight of the PEG. At very high PEG 8000 concentrations (200 g/liter) a diminution in the stimulation was observed. Among the substances in Group A (Table I), PVP produced the largest stimulation of the luciferase reaction. In the presence of PVP or PEG a marked foaming was noticed in the spent assay mixture. A control experiment using bacterial luciferase in the presence or absence of PEG or PVP revealed that the foaming did not impair light detection. Light production by this enzyme was the same with or without PEG.

The concentration-dependent stimulation of the luciferase reaction by nonionic micelle-forming substances (Group B, Table I) was markedly different from that observed with substances which do not form micelles (Group A). For example, at low Triton concentrations no stimulation was observed, but above a certain critical Triton concentration a sudden and dramatic stimulation occurred (Fig. 2). A similar concentration dependence of the stimulation was observed with the other nonionic surfactants investigated (Tween 20, lauryl maltoside, octyl glucoside).

Anionic and cationic surfactants (Groups C and D, Table I) completely inhibited the luciferase reaction at high concentrations. Inhibition diminished as the concentration of these substances in the assay buffer was progressively lowered.

In contrast to the anionic and cationic surfactants the zwitterionic surfactant "Zwittergent" (Group E, Table I) stimulated luciferase activity and the stimulation showed a concentration dependence similar to that observed with the nonionic surfactants (Group B).

Different Firefly Luciferase Preparations

In order to preclude the observed stimulation being an artifact of the particular firefly luciferase preparation, two other preparations were tested. PEG gave comparable stimulations with both of these preparations.

Wavelength of the Emitted Light

The wavelength maxima of the emitted light of the luciferase reaction in the presence or absence of various solvents were identical within experimental error and agreed with the previously published value of 560 nm (8).

Kinetics

The only apparent differences in the kinetics of light emission were observed in the presence of Triton and Tween. Figures 3A, B, and C show the time course of light emission from a control, a Triton-stimu-

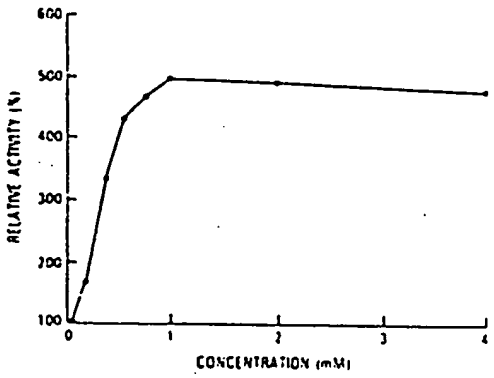

FIG. 2. Concentration dependence of stimulation of peak light emission of a firefly luciferase reaction by Triton X-100. Concentration shown is final concentration in assay tube.

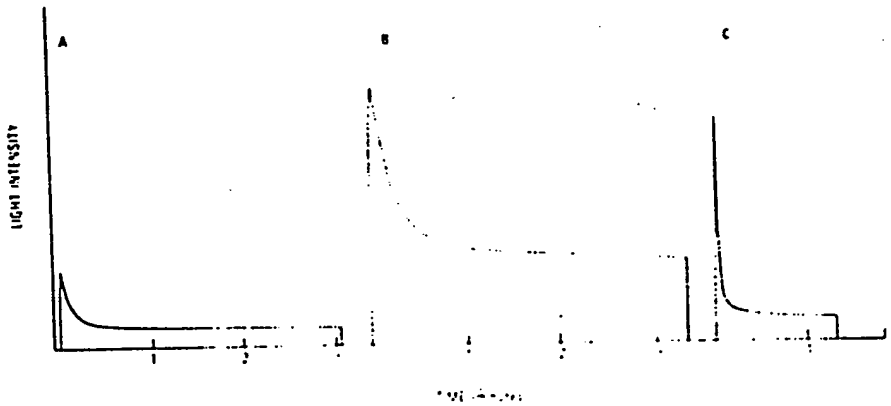

FIG. 3. Kinetics of firefly luciferase bioluminescence. (A) control; (B) in the presence of Triton (0.05 mM); and (C) in the presence of Tween (0.75 mM). Enzyme concentrations were identical in all three experiments and the recorded light intensities are directly comparable.

lated, and a Tween-stimulated reaction, respectively. Flash height was increased by Triton and by Tween, and for the latter, the time to reach peak light was more rapid than was observed with the control (Fig. 3). The decay of light emission, which reflects the rate of product inhibition, from the Triton-stimulated reaction was significantly slower than that of the control (Fig. 3A vs B). Measurement of the total light emitted during a three-minute period revealed a comparable stimulation in peak and total light emission for both the Triton and Tween-stimulated reactions.

Order of Addition of Substrates

If luciferin or luciferase is injected to initiate the reaction rather than ATP, then the Triton stimulation of peak light emission was abolished but total light emission was increased twofold. Again the decay rate was slower than that of the control.

Injections of solutions of Triton or PEG after the initial flash of light caused a slow increase in light output which peaked and then slowly decayed (Fig. 4). A similar increase in light ouput was observed with PEG.

Mechanistic Studies

The Michaelis-Menten constants (K_m) for luciferin and for ATP-Mg^{2+} in the presence of PEG and in the presence of Triton were unchanged from values obtained in the absence of these solvents. In order to determine if the Triton and PEG were unmasking additional active sites on the enzyme, titration of the active sites with L and ATP-Mg^{2+} was done in the presence and absence of these substances. In all cases one L-AMP was formed per 100,000 daltons of enzyme in agreement with previous results (9).

Another measure of the activation reaction, Eq. [1], can be determined from the rate of exchange of $^{32}PP_i$ into ATP in the presence of L. Triton caused a small (30%) stimulation of the exchange rate; however, this is not nearly as great as the observed enhancement of light emission.

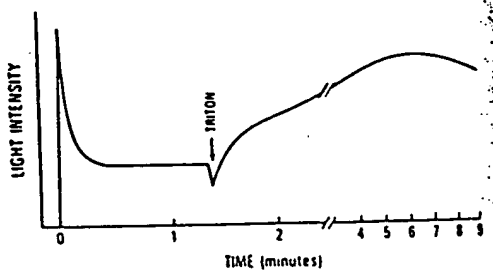

FIG. 4. Effect of a postflash injection of Triton on the kinetics of the firefly luciferase reaction. At 0 time ATP was injected into a standard luciferase assay mixture. At the point indicated, 50 μl of 4 mM Triton was injected.

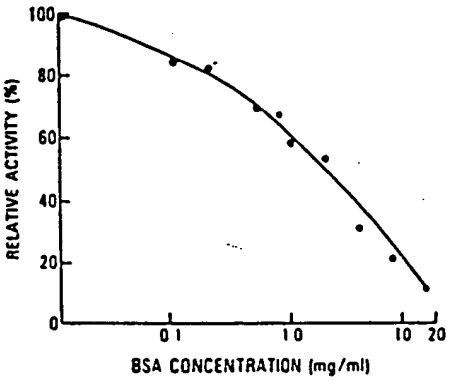

FIG. 5. Effect of BSA on PEG 8000-stimulated firefly bioluminescence. Concentration shown is final concentration in assay tube. Assay conditions are described under Materials and Methods.

Protein Effects

BSA (4 mg/ml in assay mixture) produced a twofold stimulation of peak light emission. However, BSA produced a concentration-dependent reduction in the PEG, PVP, Triton, lauryl maltoside, and Zwittergent stimulation of the luciferase reaction (e.g., Fig. 5).

Gel Electrophoresis

When luciferase is electrophoresed in 7.5% acrylamide gels in phosphate buffer, between four and five bands are seen, presumably due to aggregation of the enzyme. If the gels are run in the presence of Triton, none of the enzyme moves into the gels. Catalase, M_r 232,000 daltons, migrates into the gel in the presence of Triton. Apparently the Triton and luciferase form a large complex which is excluded from these gels. In gels containing PVP or PEG the migration of luciferase and the control (catalase) was also reduced.

DISCUSSION

The bioluminescence of the firefly luciferase reaction is stimulated by a variety of substances with differing molecular weights and chemical properties. Generally, nonionic surfactants are the most effective stimulants for the reaction and for these substances the most probable mechanism is one involving micelles. The relationship between stimulation and surfactant concentration shows the characteristic abrupt change in stimulation above a certain critical surfactant concentration which approximated to the critical micelle concentration (CMC). The observed CMC's based on stimulation of luciferase activity were—Triton X-100, 0.75 mM; Tween 20, 0.82 mM; laurylmaltoside, 0.05 mM; octyl glucoside, 9 mM: literature values are 0.12, 0.24, 0.2, and 20 mM (10), respectively.

PEG, which does not form micelles, and Triton, which does form micelles, were chosen for extensive investigation. Both of these stimulate light production between 1.5- and 5-fold. The stimulation is not due to an unmasking of additional active sites as demonstrated by the titration with L and ATP-Mg^+ in the presence or absence of the polymers. It is not due to a change in K_m's for the substrates. It is not likely that either PEG or Triton are concentrating LH_2 near the active site of the enzyme, since LH_2 is present in saturating concentrations in the experiments. The oxidative reaction is effected since there was very little increased activity in the $^{32}PP_i$-ATP exchange reaction. While the exact mechanism of the stimulation is unknown, there must be increased turnover of the enzyme in the presence of these solvents. This is apparent from the increased total light obtained and also since the quantum yield of the reaction is 0.88 it is not possible that the stimulation is due to an increased quantum yield (11). The stimulation of luciferase by PEG and Triton after the initial flash of light (Fig. 4) when the enzyme is product inhibited is compelling evidence that both PEG and Triton are increasing the turnover of the enzyme. One reasonable explanation is that the polymers bind to the enzyme in such a manner that the product molecule, which is inhibitory and normally tightly bound, is somehow more readily dissociated.

An interesting observation is that Triton does not stimulate peak height if the enzyme reaction is initiated by injecting LH_2 or enzyme. However, in both cases the total light was increased. When enzyme

is mixed with LH_2 and ATP injected, both peak light and total light are stimulated. The nonequivalence of the results when LH_2, ATP, or enzyme initiates the reaction suggests that interactions between Triton micelles and the enzyme and substrates are different and either irreversible or only slowly reversible in the heterogeneous reaction mixture. Most light is obtained from reactions in which Triton and LH_2 are mixed together, i.e., enzyme or ATP injection. Results from two-phase water/Triton experiments show that LH_2 distributes preferentially into the Triton phase. Thus interaction between enzyme and LH_2 contained in Triton micelles may be a key event in the stimulation of light output. The enzyme is known to undergo a large conformational change during reaction (12) and it is possible that the Triton-LH_2 enzyme interaction promotes this conformational change. There are several reports of binding of Triton X-100 to proteins (13) and the inhibition of stimulation by BSA suggests that BSA may compete with the enzyme for binding to Triton, thus preventing the stimulation.

The mechanism proposed for the Triton stimulation cannot operate for substances such as PEG, which does not form micelles. Although it has been shown that PEG does not bind to certain soluble proteins (14), firefly luciferase is a very hydrophobic protein and thus may be an exception (7). Thus luciferase:PEG aggregates may act in a similar manner to luciferase:Triton aggregates. Evidence from gel electrophoresis supports this hypothesis. In the presence of PEG and also in the presence of PVP, electrophoresis was impaired, which suggests the formation of high molecular weight complexes. However, an alternative explanation is that in the presence of PEG the enzyme precipitates at the top of the gel (15).

Anionic and cationic surfactants failed to stimulate the luciferase reaction. The reasons for this are obscure since positively charged micelles formed from cationic surfactants would be expected to stimulate the luciferase reaction, since all of the substrates are negatively charged.

Stimulation by the lower molecular weight substance, e.g., ethylene glycol and glycerol, is difficult to explain. Both are widely used to stabilize enzyme (16) but we are unaware of any reports of stimulations of enzyme turnover.

The rates of many organic reactions are altered by surfactants. This type of stimulation is normally explained in terms of solubilization and concentration of reactants by micelles (17). Studies of enzyme-catalyzed reactions in the presence of surfactants is less extensive (18). Both increased and decreased rates of reaction have been observed depending upon the particular enzyme and surfactant. For example, Triton X-100 stimulates the activity of glucose-6-phosphate phosphohydrolase; whereas sodium lauryl sulfate and Tween 20 inhibit the activity of this enzyme (19). An apparent stimulation of a lactate dehydrogenase-mediated reduction of tetrazolium salts by Triton X-100 has also been described (20). No detailed mechanistic interpretations or generalizations have been proposed to explain micelle effects on enzyme catalyzed reactions. In the solvent-stimulated luciferase reactions described here, solvents such as Triton and PEG stimulate enzyme activity by increasing the turnover of the enzyme but as yet the exact mechanism of this stimulation remains unknown.

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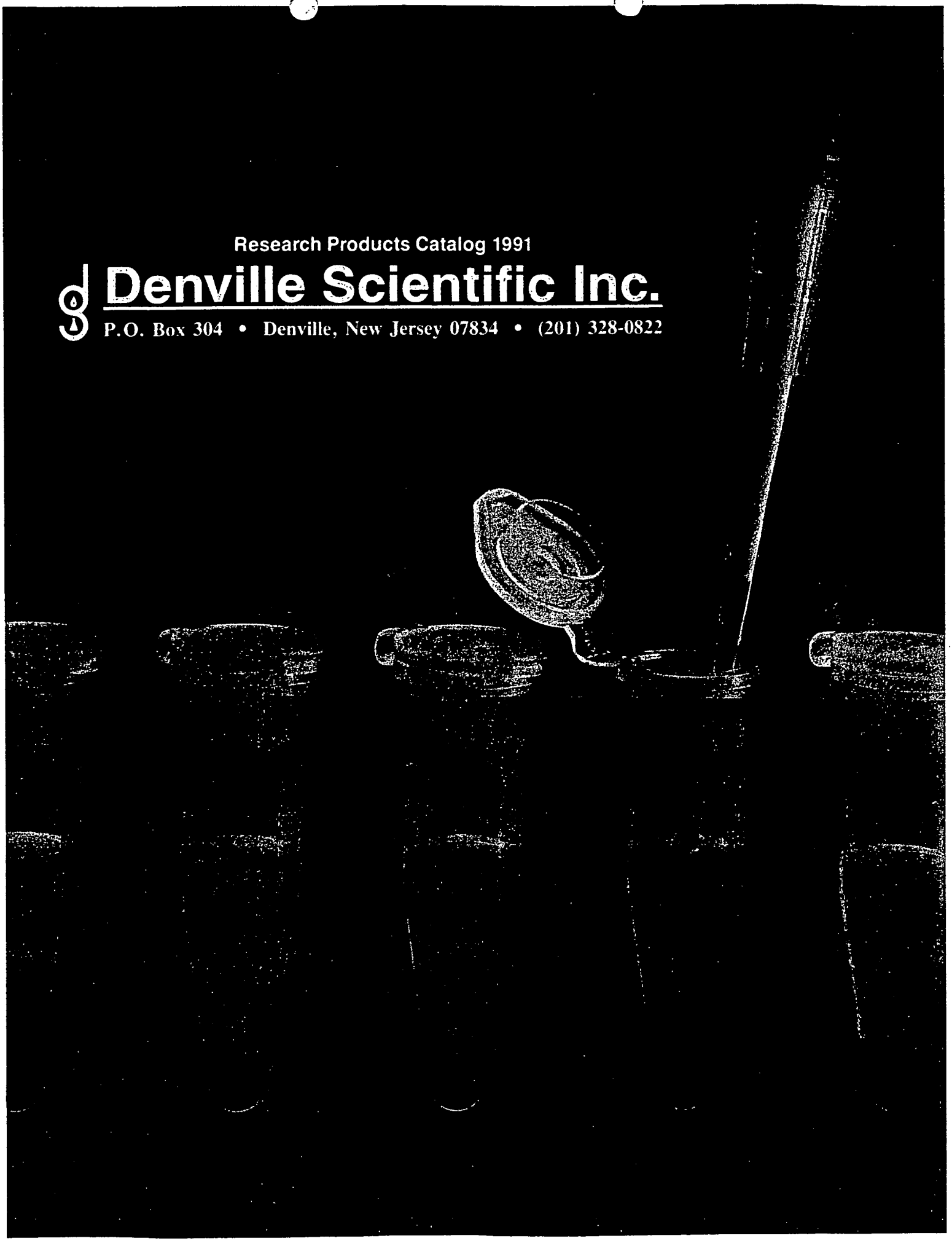

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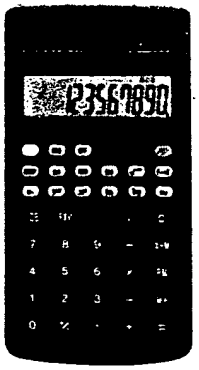

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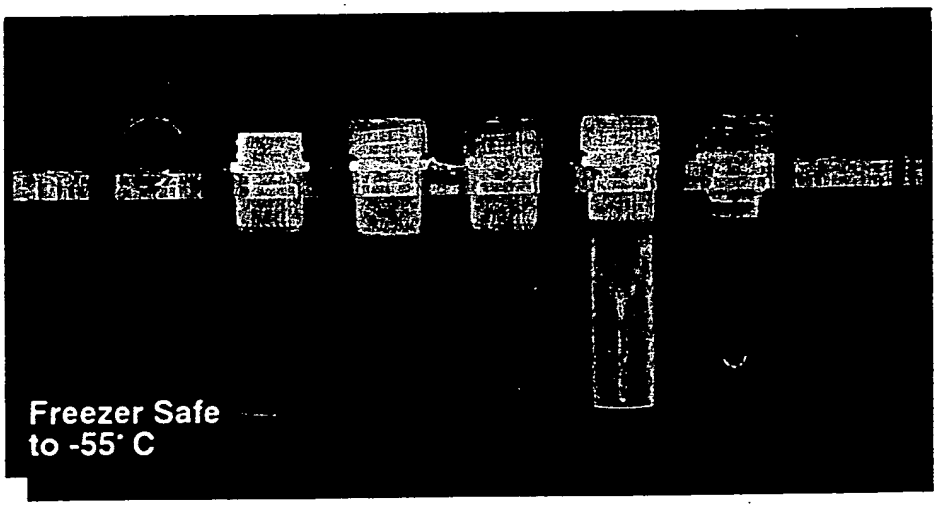
Siliconized Microcentrifuge Tubes

Our premium quality graduated tubes (shown preceding page) are also available with a prelubricated surface for applications requiring minimal binding characteristics. A special proprietary compound is used, eliminating any need for time consuming siliconization. These tubes are ideal for easy removal of DNA pellets, gene library work, precipitating restriction enzymes, lambda PHAGE procedures, DNA sequencing, or for any application requiring that samples fall directly and immediately to the bottom of the tube.
All sizes are available in rainbow pack colors and all are **RNase free.** The dimensions of the 600ul size make it compatible with major temperature cyclers.

Denville No.	Description	Pkg. Qty.	Price
C-19063	600ul, natural color	500	$18.9(
C-19065	600ul, rainbow pack	500	19.9(
C-19033	1.7ml, natural color	250	10.9(
C-19035	1.7ml, rainbow pack	250	11.9(
C-19023	2.0ml, natural color	200	11.5(
C-19025	2.0ml, rainbow pack	200	12.0(

Screwcap Microcentrifuge Tubes

New microtubes are available with or without O-rings for increased temperature range (-55° to+150° C), making them useful for freezing, boiling and PCR. Tubes and screwcaps are made of polypropylene, while the O-rings are made of a polypropylene/polyethylene blend. The **0.5ml size is compatible with all major temperature cyclers** and has uniformly thin walls and bottoms for rapid, even thermal transfer. All sizes are compatible with most popular microcentrifuges.

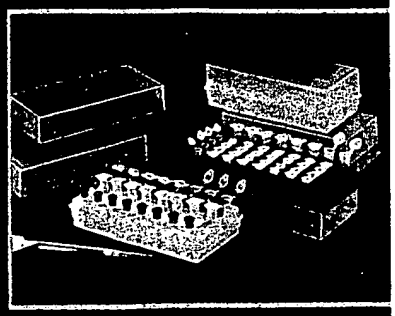


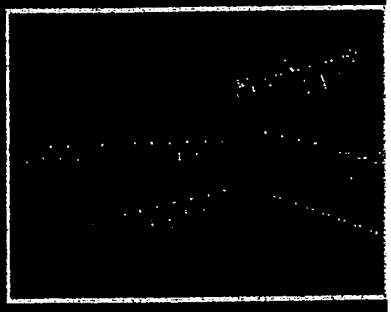
Denville No.	Packaging	Price Per 500
0.5ml		
C-19051	Tubes, without caps	$18.00
C-19050	Tubes with separate caps and O-rings	49.00
C-19050-S	Tubes with attached caps and O-rings, sterile (10 bags of 50 sterile tubes)	69.00
0.5ml Free Standing		
C-19053	Tubes, without caps	18.00
C-19052	Tubes with separate caps and O-rings	49.00
C-19052-S	Tubes with attached caps and O-rings, sterile (10 bags of 50 sterile tubes)	69.00
1.5ml		
C-19041	Tubes, without caps	18.00
C-19040	Tubes with separate caps and O-rings	49.00
C-19040-S	Tubes with attached caps and O-rings, sterile (10 bags of 50 sterile tubes)	69.00
1.5ml Free Standing		
C-19055	Tubes, without caps	18.00
C-19056	Tubes with separate caps and O-rings	49.00
C-19056-S	Tubes with attached caps and O-rings, sterile (10 bags of 50 sterile tubes)	69.00

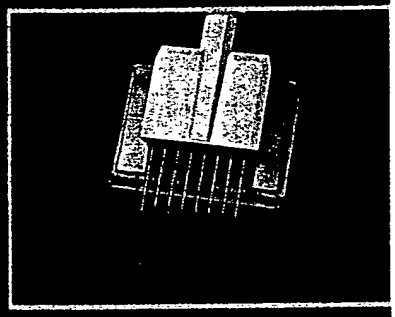
Denville No.	Packaging	Price Per 500
2.0ml		
C-19031	Tubes, without caps	$18.00
C-19030	Tubes with separate caps and O-rings	49.00
C-19030-S	Tubes with attached caps and O-rings, sterile (10 bags of 50 sterile tubes)	69.00
2.0ml Free Standing		
C-19043	Tubes, without caps	18.00
C-19042	Tubes with separate caps and O-rings	49.00
C-19042-S	Tubes with attached caps and O-rings, sterile (10 bags of 50 sterile tubes)	69.00
Screw Caps (For all sizes)		
C-19045	Caps, white, without O-rings	16.00
C-19046	Caps, white, with fitted O-rings	32.00

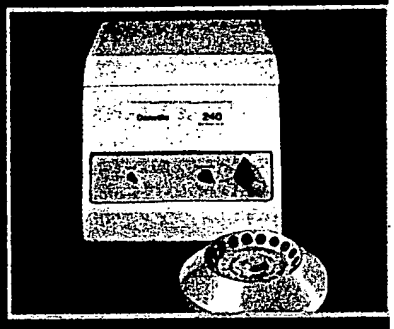
Both caps are available in red, blue, green, yellow, orange and violet. To order, add the first letter of the desired color to the end of the catalog number.

Research Products Catalog









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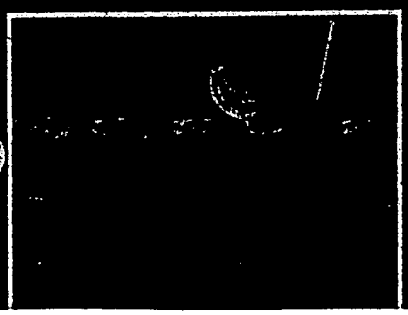
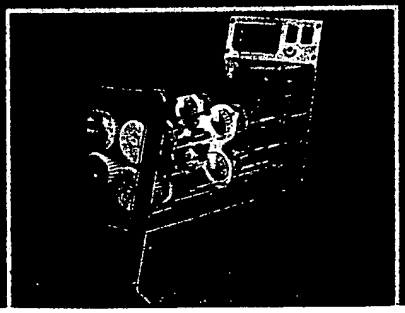
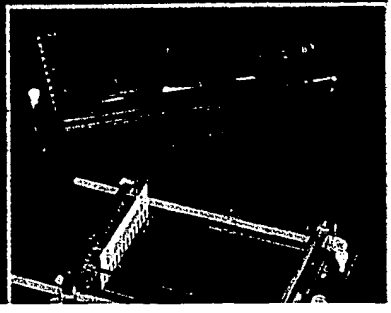
SCIENTIFIC INC.







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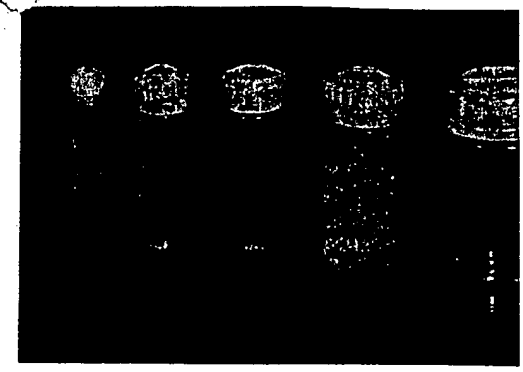
Oak Ridge Plus™ Centrifuge Tubes



- *For high speed applications*
- *Double sealing screw caps*

Oak Ridge Plus tubes combine "push-in" stopper and screw-cap technology together with 3 pressure rings to form a positive contact seal. Available in polyallomer (PA), polycarbonate (PC), and polypropylene (PP).

Cat. No. PA	PC	PP	Dimensions (mm)	Volume	Price pk/10
C-2411	C-2401	C-2421	16 x 80	10mL	$59.00
C-2961	C-2951	C-2955	25 x 92	30mL	79.00
C-3211	C-3201	C-3205	29 x 103	50mL	79.00
C-3245	C-3241	C-3243	38 x 105	80mL	109.00
C-3421	C-3401	C-3411	62 x 122	250mL*	64.00/6

*The 250mL size is a flat bottom bottle, is not Oak Ridge type and is supplied in packs of 6.

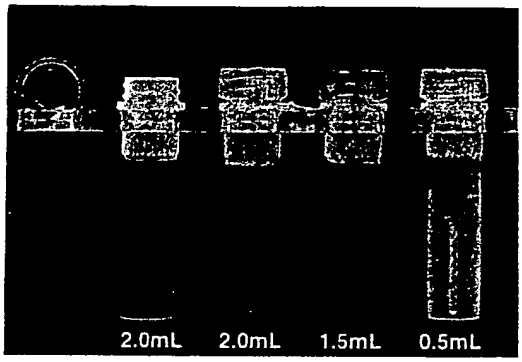
Flip-Top Centrifuge Tubes

- *For high speed and ultra rotors*

These autoclavable polyallomer tubes have an integral hinged cap and are designed to withstand repeated high speed use.

Cat No.	Dimensions (mm)	Volume (mL)	Max. rpm	Price pk/10
C-2036-5	11 x 40	2.5/1.4	30K	$13.75
C-2036-6	11 x 60	4.4/2.4	30K	27.50
C-2036-7	13 x 64	6.5/3	26K	29.70
C-2036-8	16 x 76	13.5/8	25K	33.00
C-2036-9	25 x 89	38.5/20	28K	38.50
C-2037-0	29 x 103	50/37	24K	49.50
C-2037-1	38 x 102	100/51	20K	58.30

Screw-Cap Microcentrifuge Tubes



- *-70 to +130°C temperature range*
- *Etched writing surface on one side*
- *Light blocking amber tubes available*

Cat No. Conical	Cat. No. Free Standing	Description/ Packaging	Price pk/500
0.5mL			
C-19051	C-19053	Tubes without caps, 0.5mL	$18.00
N.A.	C-19053-A	Tubes without caps, amber color	18.00
C-19050	C-19052	Tubes with separate caps and o-rings	49.00
C-19050-S	C-19052-S	Sterile tubes with attached caps and o-rings (10 bags of 50 sterile tubes)	69.00
1.5mL			
C-19041	C-19055	Tubes without caps, 1.5mL	18.00
C-19040	C-19056	Tubes with separate caps and o-rings	49.00
C-19040-S	C-19056-S	Sterile tubes with attached caps and o-rings (10 bags of 50 sterile tubes)	69.00
2.0mL *(2mL free standing tubes are graduated)*			
C-19031	C-19043	Tubes without caps, 2.0mL	18.00
N.A.	C-19043-A	Tubes without caps, amber color	18.00
C-19030	C-19042	Tubes with separate caps and o-rings	49.00
C-19030-S	C-19042-S	Sterile tubes with attached caps and o-rings (10 bags of 50 sterile tubes)	69.00
Screw Caps and Racks *(for all sizes)*			
C-19045		Caps, natural color without o-rings	16.00
C-19046		Caps, natural color with fitted o-rings	32.00
C-19047		Caps, amber without o-rings	16.00
C-19048		Caps, amber with fitted o-rings	32.00
R-7872-NT		"No-turn" rack for 40 tubes	39.00

C-19045 and C-19046 caps are available color coded red, blue, green, yellow, orange, violet and assorted. To order, add the first letter of the desired color to the end of the catalog number.

Class | ISSUE CLA

SERIAL NUMBER	FILING DATE	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
60/001,081 PROVISIONAL	07/12/95				

APPLICANTS

RICHARD SKIFFINGTON, EVERETT, MA; ELIEZER ZOMER, NEWTON, MA.

CONTINUING DATA********
VERIFIED

_____

FOREIGN/PCT APPLICATIONS********
VERIFIED

_____

FOREIGN FILING LICENSE GRANTED 09/11/95 ***** SMALL ENTITY *****

Foreign priority claimed / 35 USC 119 conditions met	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
☐ yes ☐ no / ☐ yes ☐ no	→	MA	3			$75.00	95-72

Verified and Acknowledged _____ Examiner's initials

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OSTERVILLE MA 02655-0901

TITLE

TEST KIT AND METHOD FOR THE DETERMINATION OF ATP

BAR CODE LABEL

U.S. PATENT APPLICATION

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT
60/001,081 PROVISIONAL	07/12/95		

APPLICANT

RICHARD SKIFFINGTON, EVERETT, MA; ELIEZER ZOMER, NEWTON, MA.

CONTINUING DATA********
VERIFIED

_____

FOREIGN/PCT APPLICATIONS********
VERIFIED

_____

FOREIGN FILING LICENSE GRANTED 09/11/95 ***** SMALL ENTITY *****

STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS	FILING FEE RECEIVED	ATTORNEY DOCKET NO.
MA	3			$75.00	95-72

ADDRESS

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OSTERVILLE MA 02655-0901

TITLE

TEST KIT AND METHOD FOR THE DETERMINATION OF ATP

This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application which is identified above.

By authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

Date Certifying Officer

PATENT APPLICATION SERIAL NO. 60/001081

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

200 LG 08/17/95 60001081
1 214 75.00 CK

PTO-1556
(5/87)

Approved for use through 04/11/98. OMB 0651-0037
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION COVER SHEET

60/001081

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

Docket Number	95-72	Type a plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Skiffington	Richard		33 Linden Street Everett, Massachusetts 02149 MA
Zomer	Eliezer		374 Kenrick Street Newton, Massachusetts 02158 MA

TITLE OF THE INVENTION (280 characters max)

TEST KIT AND METHOD FOR THE DETERMINATION OF ATP

CORRESPONDENCE ADDRESS

Richard P. Crowley, Attorney-at-Law, P.C.
P.O. Box 901, 901 Main Street
Osterville

STATE	MA	ZIP CODE	02655-0901	COUNTRY	USA

ENCLOSED APPLICATION PARTS (check all that apply)

x	Specification	Number of Pages: 2	x	Small Entity Statement	
x	Drawing(s)	Number of Sheets: 3	x	Other (specify)	Postcard, Check

METHOD OF PAYMENT (check one)

x	A check or money order is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE AMOUNT ($)	$75.00
x	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: 03-3816 (for fee deficiency only)		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[x] No.

[ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Richard P. Crowley Date 07/12/95

TYPED or PRINTED NAME Richard P. Crowley REGISTRATION NO. (if appropriate) 19,745

[ ] Additional inventors are being named on separately numbered sheets attached hereto

PROVISIONAL APPLICATION FILING ONLY

3

75-214 A/provisional
60/001081

TEST KIT AND METHOD FOR THE DETERMINATION OF ATP

The present invention concerns a portable pocket-swab type test kit and method for the detection of the presence of ATP on surfaces in water and other biological fluids or foods, and also can be used to detect marker enzymes and/or antibiotics.

The device consists of a plunger with a swabbed end, which is inserted through a cylinder into a microtube, said microtube consisting of a microbialysis solution and an ATP stabilizer, a buffer optimized for luciferin-luciferase reaction, and a luciferin-luciferase reagent tablet individually packaged in plastic cylinders sealed with aluminum under optimum conditions which give the test kit excellent stability, at the base of the microtube.

The test is performed by removing the seal securing the plunger to the cylinder, and removing the plunger. After swabbing/sampling the effected area, the plunger and sample are reinserted into the cylinder and into the microtube. The cylinder has three indented markings on the outside. When the plunger is reinserted into the cylinder, it is depressed to the second mark, and the plunger is twirled twice, breaking into the first cylinder (reagent A). The plunger is then depressed to the third mark and twirled twice more, breaking into the second cylinder (reagent B). The plunger is then depressed fully, breaking into the last chamber (reagent C) and is then twirled. The plunger is then withdrawn to the first mark, moistening the reagent tablet (reagent C) at the bottom of the microtube.

The microtube is detached from the cylinder after removing the

aluminum seal. The microtube is then covered with the sticker cap and counted.

Each test kit is fully packaged all in one device, including the reagents, which greatly simplifies the test, making it user-friendly. The test utilizes simple steps which are controlled by the plunger, and has aluminum foil that separates the various compartments. It eliminates the need to prepare reagents, and no pipettes or dispensers are needed. This device eliminates operational mistakes due to inaccurate pipettes. Since all the reagents, liquid and tablet, are individually packaged in plastic cylinders which are sealed with aluminum, then under optimum conditions, the test kit has excellent stability, with an expectation of over two month's stability at room temperature. This device can be used in any place in a processing plant without restrictions.

Three sheets of drawings fully explaining this test kit and the method are enclosed.

PATENT

Attorney's Docket No. 95-72

Applicant or Patentee: R. Skiffington et al

Serial or Patent No.: 0 /

Filed or Issued:

For: TEST KIT AND METHOD FOR THE DETERMINATION OF ATP

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c))—SMALL BUSINESS CONCERN

I hereby declare that I am

☐ the owner of the small business concern identified below:

☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN Charm Sciences, Inc.

ADDRESS OF CONCERN 36 Franklin Street
Malden, MA 02148

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed, to and remain with the small business concern identified above with regard to the invention, entitled TEST KIT AND METHOD FOR THE DETERMINATION OF ATP

by inventor(s) Richard Skiffington and Eliezer Zomer

described in

☒ the specification filed herewith.

☐ application serial no. 0 / _____, filed _____.

☐ patent no. _____, issued _____.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: *Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).*

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Richard J. Long

TITLE OF PERSON OTHER THAN OWNER Clerk

ADDRESS OF PERSON SIGNING 36 Franklin Street
Malden, MA 02148

SIGNATURE Richard J. Long Date July 12, 1995

60/001,081

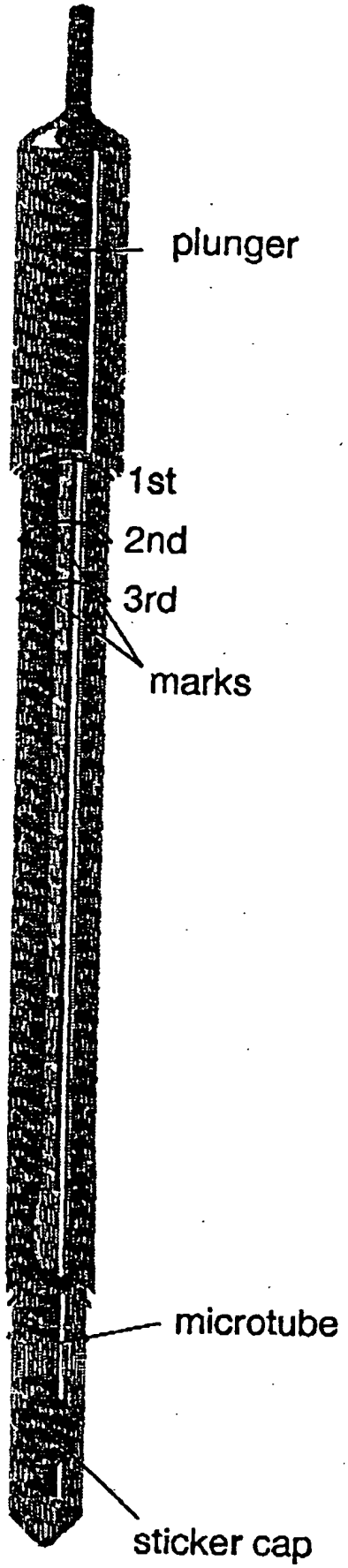


FIG 1

60/001,081

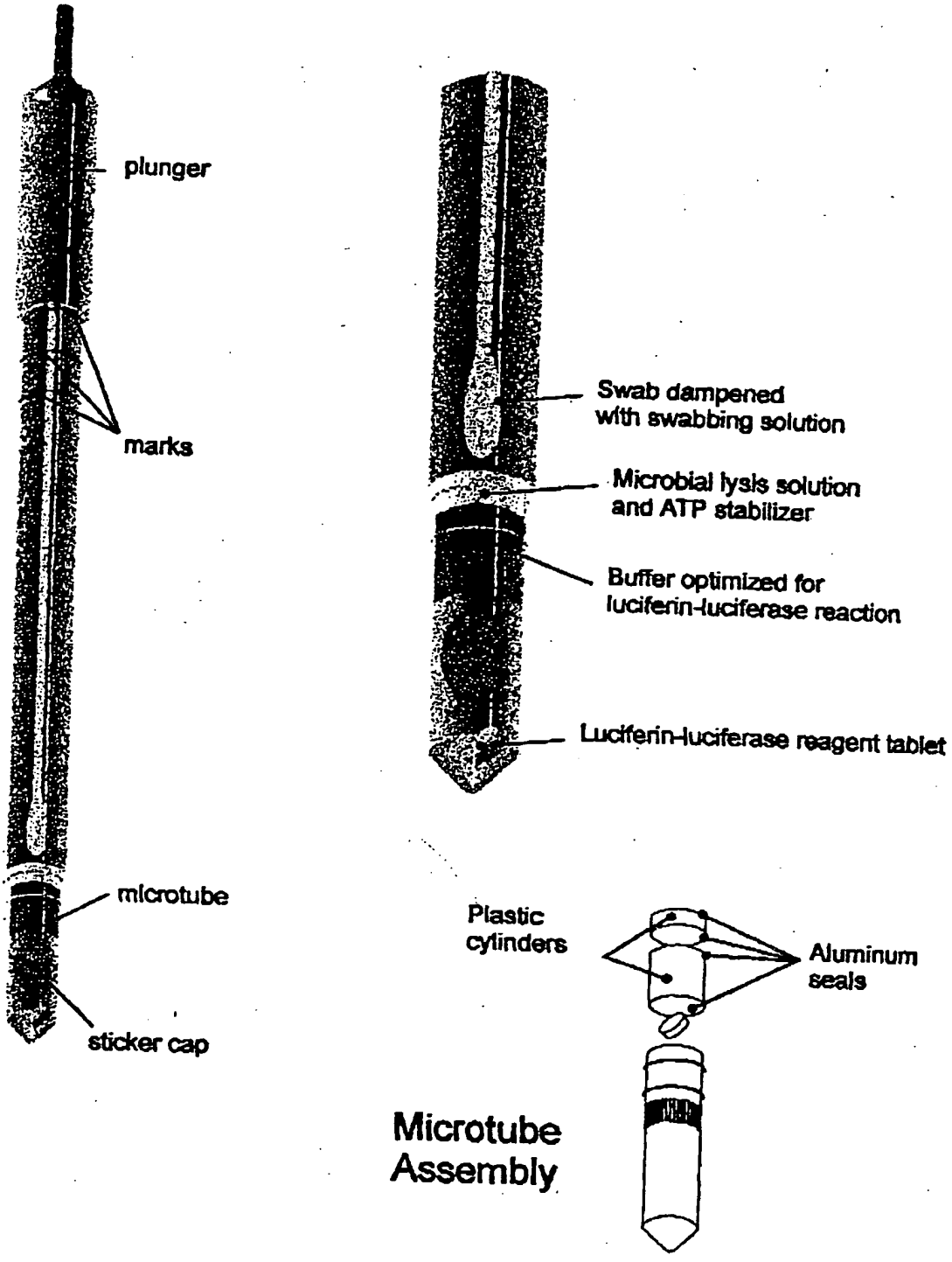
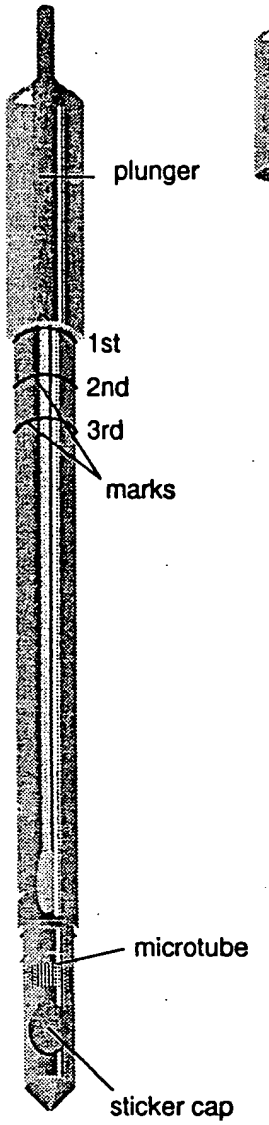


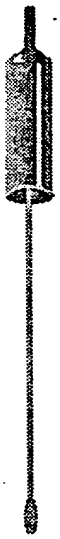
FIG 2

60/001,091

PocketSwab™ Procedure



1 Remove seal, withdraw plunger. Swab area.



2 Reinsert plunger. Depress to second mark. Twirl twice.

3 Depress to third mark. Twirl twice.

4 Depress fully. Twirl until drop moistens tablet. Withdraw to first mark.

5 Remove seal and detach microtube. Cover with sticker cap and Count.

CHARM SCIENCES INC.
36 FRANKLIN STREET MALDEN MA 02148 USA
800 343-2170 FAX 617 322-3141
Nothing works like a Charm.

PKSWB01

FIG 3

60/001081

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Richard Skiffington et al

Docket No. : 95-72

Filing Date : July 12, 1995

Title : TEST KIT AND METHOD FOR THE DETERMINATION OF ATP

Certificate of Express Mail under 37 C.F.R. 1.10

I hereby certify that the enclosed "Provisional Application cover Sheet, Specification, Drawings, Verified Statement Claiming Small Entity, check and postcard are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date shown below and is addressed to the Commissioner of Patents and Trademarks, Box Provisional Patent Applications, Washington, D.C. 20231, under **Express Mail Label No. TB579874405 US.**

J. Shanahan

Jane E. Shanahan

Date: July 12, 1995

Enclosure: Provisional Application Cover Sheet
Specification
Drawings
Verified Statement Claiming Small Entity
Postcard
Check

PTO/SB/58 (07-03)
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In re Application of

Application Number	Filed
60/001,081	7/12/95

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Paper No. 2

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United States Patent Application Publication No. _____, page, _____ line _____,

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Michael O. Linton
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9/4/02
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1ST EXAMINER Artus

2ND EXAMINER

APPLICATION NUMBER	TYPE APPL	FILING DATE MONTH	DAY	YEAR	SPECIAL HANDLING	GROUP ART UNIT	CLASS
[redacted]081	9	07	12	95	0		

TOTAL CLAIMS	INDEPENDENT CLAIMS	SMALL ENTITY?	FILING FEE	FOREIGN LICENSE	ATTORNEY DOCKET NUMBER
		2	--75	Y	95-72

CONTINUITY DATA

CONT CODE	STATUS CODE	PARENT APPLICATION SERIAL NUMBER	PCT APPLICATION SERIAL NUMBER	PARENT PATENT NUMBER
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PCT/FOREIGN APPLICATION DATA

FOREIGN PRIORITY CLAIMED	COUNTRY CODE	PCT/FOREIGN APPLICATION SERIAL NUMBER	FOREIGN FILING DATE MONTH	DAY

60/081081

PATENT APPLICATION

60001081

APPROVED FOR LICENSE

INITIALS AJR 09547

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Date Entered or Counted			Date Received or Mailed
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Class	ISSUE CLA

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60/007,585 PROVISIONAL	11/27/95				

APPLICANTS

RICHARD SKIFFINGTON, EVERETT, MA.

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Foreign priority claimed / 35 USC 119 conditions met	Verified and Acknowledged	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
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TITLE

TEST APPARATUS SYSTEM AND METHOD FOR THE DETECTION OF TEST SAMPLES

BAR CODE LABEL

U.S. PATENT APPLICATION

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT
60/007,585 PROVISIONAL	11/27/95		

APPLICANT

RICHARD SKIFFINGTON, EVERETT, MA.

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TITLE

TEST APPARATUS SYSTEM AND METHOD FOR THE DETECTION OF TEST SAMPLES

This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application which is identified above.

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PATENT APPLICATION SERIAL NO. 60/007585

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FEE RECORD SHEET

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(5/87)

PROVISIONAL APPLICATION COVER SHEET

60/007585

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

Docket Number	95-72	Type a plus sign (+) inside this box →	+

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TITLE OF THE INVENTION (280 characters max)

TEST APPARATUS, SYSTEM AND METHOD FOR THE DETECTION OF TEST SAMPLES

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STATE	MA	ZIP CODE	02655-0901	COUNTRY	U.S.A.

ENCLOSED APPLICATION PARTS *(check all that apply)*

[X]	Specification	*Number of Pages*	40	[ ]	Small Entity Statement
[X]	Drawing(s)	*Number of Sheets*	2 (two)	[ ]	Other (specify)

METHOD OF PAYMENT *(check one)*

[X]	A check or money order is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE AMOUNT ($)	$ 150.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No.

[ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Richard P. Crowley

Date 11/27/95

TYPED or PRINTED NAME Richard P. Crowley

REGISTRATION NO. *(if appropriate)* 19,745

[ ] Additional inventors are being named on separately numbered sheets attached hereto

PROVISIONAL APPLICATION FILING ONLY

60/007585

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Richard Skiffington et al

Docket No.: 95-72 Group No.:

Filed: **November 27, 1995** Examiner:

For: TEST APPARATUS, SYSTEM AND METHOD
FOR THE DETECTION OF TEST SAMPLES

Commissioner of Patents and Trademarks

Washington, D.C. 20231

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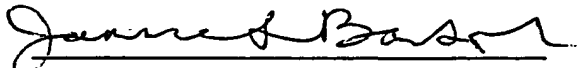

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Test Apparatus System and Method for the Detection of Test Samples

Description

Reference to Prior Applications

This application is a continuation-in-part of provisional patent application 60/001,081 filed July 21, 1995, hereby incorporated by reference. Corresponding design continuation-in-part application, Docket No. 95-99, filed ------ is also hereby incorporated by reference.

Background of the Invention

It is widely desired to provide for a rapid assessment efficient and test for the detection of various test samples from materials or surfaces. Various test apparatuses and test methods have been developed for that purpose. For Example, it is widely desirable to determine or to test through quantitative and qualitative body fluids, such as blood, urine, milk and the like, as well as food, such as meat processing, fruit, vegetables, and to detect for alkaline phosphatase, shell, processes meats, drugs, and antibiotics, such as for example, sulphur drugs organophosphates carbamates and active metabolites for various bacteria and pathogenic combinations, either in materials or on the surface of materials or both.

The earlier stated example, for illustrated example only for the detection characterization of qualitative and qualitatively through the employment of a color change or a bioluminescent test, for the detection of the alkali phosphatase, such as for example, the detection of ATP on or in materials most desirable for providing a measure of immunoeffectiveness, so that a rapid determination can be determined if a process or surface are adequately clean and free of the alkaline phosphatase or otherwise so that

collective or disinfection action can be instituted. Typically, the reaction of ATP is by bioluminescence assay, which is a standard test which will detect food residue, bacteria, yeast, mold, by measuring the ATP, so in the opinion of a test, for example, on the surface of the material, such as by non-laboratory or out-of laboratory or in field locations, the activating the test sample in the presence of test reagents, and then later employing a luminometer to determine test results, which can be prepared with a controlled sample or controlled requirement.

The detection, for example, for illustration of a phosphatase, like ATP, may be formed in a dimensional plate-counting test and method. However, such a test is time consuming and requires laboratory trained personnel for the present commercial tests are generally directed to a bioluminescence, which ordinarily takes less than five minutes and employs premeasured and prepackaged separate test reagents and employs a luminometer to detect test results. Generally, a portable luminometer, which are checked for use in the field. and the use of test containers, such as various test tubes or plates. The concentration of the phosphatase has been determined by measuring or counting of the luminescence determined by the reaction mixing with the test sample, and comparing the count against certain accepted control standards or a threshold of a one of a control standard.

There are various ATP tests available in the field, and one luminescent ATP monitoring test in present use is described in "The Handbook of ATP-hygiene Monitoring" by Bio-Orbit of Oy of Turku, Finland, while another luminescent ATP hygiene monitoring test in use is called the Charm ABC Swab Test, sold by Charm Sciences, Inc., of Malden, Massachusetts, both of which test and literature are hereby incorporated by

reference.

Another portable swab type device for use in an ATP bioluminescent test for measuring cleaning effectiveness is distributed under the mark lightning swab device by Idexx Laboratories of Westbrook, Maine, Lightning is a trademark of Idexx. The lightning device consists of a unique swab designs, which contains a unit dose reagents in a used with a portable luminometer, which allows the storage of thousands of test points. and employs an elongated tube with a cover on it at one end and an elongated, extended premoistened wetting agent, and a premoistened swab, and with such end containing a buffer in a bulb, while the opposite chamber end, which the test results are read, comprises an ampule, for example, containing a luciferin and luciferase material, which with a filter material separating the read chamber form the buffer end. The swab is removed form the tube and is used to obtain a test sample form a surface to be tested for ATP, and then the swab is reinserted within the tube. The one end, that is, the cover end of the device is then met and squeezed to force out a buffer solution while the opposite ends containing the glass ampoule with the reagents, such as t he luciferin derivative and luciferase, is crushed by the user so that the buffer and the crushed luciferin-luciferase test reagents are then admixed within the tube with the wetting agent to form the reaction mixture which would provide for the appropriate bioluminescence. When the read chamber at one end is removed and inserted into and read by the portable luminometer. Thus, the lightning device provides a swab-type test probe requiring the bending and squeezing of one end and the crushing of another end, of the device and then the admixture prior to inserting in the read chamber and then reading the test results.

The Bio-orb, the Charm ABC Swab Test and the Lightning

swab device are all mixed together with their test methods and literature and equipment area all hereby incorporated by reference.

It is thus desirable to provide for a new and improved test apparatus, system and method adapted for use with a wide variety of known and unknown test methods for the detection of test materials on a material or on a surface. The improved test apparatus is greatly simplified in structure and is effective in use, eliminates possible operational mistakes by personnel in the field, does not require separate pipettes and test tubes, does not provide for the crushing of glass ampoules, provides excellent separate stability of test reagents which may be employed with the test results by specifically prepackaging the reagents, so that the test apparatus may be stored for long periods of time, and is particularly adapted for in-filed or out of the laboratory testing by unsophisticated personnel, as well as the use by laboratory personnel and further and importantly may have the test results determined by using the entire test results in one end thereof, or removing one end of the test unit for testing in a test instrument, which may be, for example, a visual change of color in some tests, a use of a portable luminometer, for the use of other types of test instruments including radioactive detection devices neither alone or in any combination. The improved test device is particularly adaptable as being a disposable, inexpensive, transparent, plastic pocket-type test apparatus.

Summary of the Invention

The present invention concerns a test apparatus and a test system employing the test apparatus, and test method employing the test apparatus and system, and in particular is directed to a color change or luminescent type test for the detection of test samples from the material or raw material

surfaces, by employing known test techniques.

The invention comprises a test apparatus, composed of a sample unit and a test unit which sample and test units may be generally longitudinally aligned, and which may be integral or may be disposed for the removable detachment of the test unit by the user, and which test apparatus is employed for the detection of the qualitative or quantitative, or for any analytical test of a test sample from or on a material or on a material surface. The test apparatus comprises a sample unit having ap robe means having a first and second end, with the first end adapted to obtain a test sample and is used to be collected from or on a material, and generally would comprise a probe-type means at on end, by which a test sample may be collected, and a sterile chamber having a first and second end and adapted to receive and retain therein prior to use, and optionally after use, the said probe means, and having a cover for the first end of said chamber to seal the chamber of the chamber. The sample unit also includes means to retain said probe means within said chamber prior to use; that is, to render the apparatus sterile prior to use, and without indiscriminate movement of the probe means within the chamber. The sample unit also includes a probe positioning means, comprising a plurality of selected identification positions between the probe means and the chamber in order to identify the position of the probe means, and particularly the first end of the probe means, the test apparatus, such as within said chamber or within said test units, both before an dafter use. The sample unit also includes moving generally longitudinally removal of the first end of the probe means, in relationship to said chamber, typically over or within said chamber, for use to selected or preselect identification positions as required in the particular test method.

The test apparatus also importantly includes a test unit attached to a sample unit, generally longitudinally aligned and attached to the sample having a reagent housing, which is generally transparent so that the luminometer or visual observation may be made, and with a bottom, and having a first and second end, the first end attached to said second end of the chamber in the sample unit, and the housing adapted for use alone or integrally with the test apparatus, so that the test results may be reserved in the reagent housing, or the reagent housing may be detached and used in a test instrument, or the entire apparatus, together with the reagent housing, employed in a test instrument such as a luminometer or other instruments for the detection of the test sample.

The test unit also includes a test sample reagent means, which is preselected depending on the desired test to be carried out, and when one or more tests may be carried out alone or in any sequence as desired, with the test reagent means designed to contact the test sample collected form the material or its surface. The test sample or reagent means generally comprises at least on sealed reagent package containing a test reagent, which may be solid, liquid powder or substantially any combination thereof, and there may be and usually is a plurality of separate sealed reagent package, depending on the particular test method selected for the test sample. The test sample reagent means is characterized as being adapted, arranged and constructed so as to be penetrated by the longitudinal movement of the first end of the probe means to a selected identification position so as to permit the admixture or combination reaction or otherwise contacting the test sample on the probe means, and the one or more reagents which have been released form the sealed reagent action of the regent means.

Generally, the test sample reagent means is characterized by a puncturable foil seal or membrane, which is adapted to be penetrated by the longitudinal movement of the first end of the probe means, or by other means after collection of the test sample by the probe means, and with the one end of the probe means moved to a selected identification positions, so as to generally sequentially, and intermittently puncture the sealed reagent package in the desired sequence if desired. The puncturing at progressive, selected identification positions, usually which positions are marked on the outside of the chamber for easy observation by the user. In some test methods, no desired or required, sequentially contacting of test reagents is desired, and generally the reagents are merely separated in order to provide for elongated or better storage life, provided merely for the convenience of the test. Generally, three or four or more reagents are employed, and would include, for example, at least one liquid reagent, wither water or a buffer solution or a neutralizing solution, and then one or more powdered or tablet type solutions, so that as the test sample on the probe means is pushed down or comes in contact with each of the selected punctured reagents.

The test apparatus, as described thus, containing the sample and test units, are particularly where they are composed of any elongated plastic or thermoplastic transparent plastic tube having a cover with an elongated probe exposed within the sterile chamber of the tube, and a transparent in an of the other and containing the prepackaged test reagents, is well adapted for use in the field by generally untrained personnel to obtain test samples form or on materials and to provide for the testing of the samples, in or on a wide variety of materials, wherein the test apparatus is composed of a disposable, transparent tube

material that is easily carried by a user in the field, and is usually disposed of in toto, or where the test unit is removed from the bottom and then is sealed, may be used in a portable luminometer, which thus makes disposal of the test apparatus quite easy, without undue contamination of the atmosphere.

Generally, the probe means comprises an elongated element secured at second end to said cover, and which cover is mounted over the one end of the chamber, typically slidably but mostly removable helical or other longitudinal movement in the one end of the chamber, and the probe means contains a test sample collection material secured at the one end, such as, for example, a fibrous type material such as a cotton swab, which may be if desired, premoistened such as by an aqueous wetting solution, or with other color indicators, dyes reagents or test reagents, or merely may contain chemicals which bind to the material to which the test is directed. Generally, the first end of the probe means is liquid moistened, such as by water or a wetting agent solution, particularly when it is used for the determination of phosphates on materials or surfaces, to determine for hygiene cleanliness, in order to aid in the collection of the test sample on the surface.

The test apparatus as provided to the user with a sample and test units together with the probe means within the sterile chambers of the sample unit, and generally the probe means is in position, so that it does not longitudinally move until after the retention for the test sample by the user, and then moved to the selected identification positions. Therefore, optionally and preferably the test apparatus would include some means to retain the probe means in a selected non-use position, prior to use by the user, such as the use of an adhesive tape wrapped about the one end of the cover

and the chamber, which is user removable and replaceable, and the use of an easily disposable, breakable adhesive, or the use of a heat shrinkable material, such as a plastic material which may be shrunk around the one end of the cover and the chamber or the entire test apparatus to render it sterile or other such means to preposition the probe means with in the sterile chamber prior to use.

The test apparatus includes probe position means, in relationship between the sterile chamber and the one end of the cover containing the probe means. One acceptable and preferential method of use. The probe position means generally would comprise any type of means by which the one end of the cover containing the probe means is moved longitudinally in relationship to the test unit which contains the reagent means. Thus, for example and preferably, the generally intermediately, though not required, the chamber may contain a series of spaced-apart, generally parallel identification marks, either marked by colors or number or both, or by some identification means, whereby the bottom portion of the cover containing the probe means and prior to the removal of the retaining means is prepositioned. The test instructions will then permit the obtaining of the test sample using the probe means on a material or a surface, and reinserting the probe means within the chamber, through a selected, usually first identification mark, or in the one end of the probe chamber, and does not extend beyond the second end of the chamber, that is, above the test unit. The probe position means then provides for the longitudinal movement of the cover means with the cover with the test probe, to say, a second position, third or fourth or multiple positions, whereby the one end of the probe means then punctures the membranes of the respective reagent test means in the test unit. Thus providing for

contact of the test sample of the probe means with the test reagents so punctured, so that all of the test samples or reagents are then contained within the test unit at the one end of the test apparatus. Generally, the final probe position means is such that all of the test reagent unit means have been punctured down at the one end, and the probe means is disposed within the test unit, and then may be twirled to ensure good contact, and then withdrawn to the original non-use position. Typically, within the chamber so that the test apparatus, the sample unit, or the sample and test unit together may be readily disposed of.

The position probe means should be well-marked and typically uncomplicated, so that the probe position means may be easily understood and used by people in the field.

The means to move the one end of the probe means within the cover may vary; so long as the probe means is moved longitudinally within the chamber, and from the one end of the chamber into the test unit. For example, by the employment of merely the slidable longitudinal movement when the cover is placed in a snug, close-fitting sliding position over the one open upper rend of the chamber of the sample unit, or where there are helical or spiral grooves placed on the inside of the cover, or on the outside of the chamber unit or both, to provide for the spiral movement to a selected probe position means, or where merely bumps or other means are employed so that the user may be moved easily to the selected positions. Of course, it ia also recognized that where there is only a test sample at the end of a probe and only one reagent, it may well be than no probe position means are required, other than for use or non-use, and the probe merely, after test sample is placed in the chamber, and merely longitudinally moved downwardly to contact a single reagent to force the reagent then to contact the test sample

directly into the test unit for observation, though this would indicate the use of a very simple test method, and typically would not lend itself, for example, to typical bioluminescent type method for determining phosphatase, or for the use of beta-lactams in processing of meat for determining sulfur or drug residues or organophosphate residue on products.

In another embodiment, the test apparatus may comprise a single tube with a cover, wherein the entire test apparatus, after the test sample on the longitudinal movement of the probe means, is employed in its entirety in determining the test results, that is, the test unit is not either made or detached or removable from the one end of the sample unit, but is for example, securely attached thereto by being integrally molded therewith. In such a situation, the test unit at the one end can still be inserted into a luminometer, or other test instrument, for the color change affected by the test results noted. Thus, as desired, the entire test apparatus can be disposed of in an effective and environmentally non-toxic manner. In another embodiment, which will be illustrated, the test unit at the one end of the test apparatus, can be detachably removed, thereto employing threads, or slidable fit, or a weakened mechanical section or other means, or merely just taping the units together, so that after movement of the probe to selected identification positions, then the removal of the probe means to the non-use position, the test unit at the one end of the test apparatus may be easily twisted or removed by the user, and would then contain therein the test sample s of the various reagents, in that mixture. In this particular method of operation and structure, the test unit which occupies only a small volume at one end, may then be detached and inserted, for example, into a portable luminometer, so this test method

lends itself quite readily to the use of portable test instruments and use in the field. Where this test method is employed it is often desirable to provide a means to seal the one open end of the test unit after removal from the test apparatus. This can be accomplished by a variety of means; for example, by employing a screw-type or plug-in type cap secured to the test apparatus, or by more conveniently by using a removable adhesive detachable seal, for example, which may be secured to the test apparatus and readily removed by the user after detachment of the test unit, and then placed over the open end of the test unit and wrapped around to cap the open end of the test unit. Such a seal, for example, may comprise but not be limited to, a non, patentable aluminum foil, which is adhesively sealed on one side, or any other means to cape seal or otherwise secure the one open end of the test unit.

It is sometimes desired to provide, rather than a generally cylindrical tube for the test apparatus, a tube wherein the plastic is flexible, particularly toward the one of the test unit, so that a user may squeeze the one end of the tube generally intermediate the test unit and the sample unit, so as to insure the test sample on the probe means is squeezed out together, for example, with the premoistened liquid and contacts the test reagents fully before the one end of the probe means is withdrawn into the chamber.

The reagent housing which is used typically is transparent, particularly where a visual observation is desired; however, it is recognized that the reagent housing may be non-transparent, particularly where the particular test to be carried out does not require transparency of the housing. The test sample reagent means, which is placed generally in the test unit, is adapted to be punctured by one end of the probe, and provides powdered, liquid, tablet or

suspensions or one or more or a combination of chemicals, materials and reagents as desired by any particular test. A typically, the test reagents would on general task comprise from say two to five separate sealed reagent packages, at least one of which package would be a liquid package, such as a water or buffer solution or a saline solution, and generally it is desirable to place at least one of the test sealed reagent package individual dye, or in each package, so that to be insured that the test probe probes each package and that the dye is present in the reagent housing. Generally, for example, the sealed reagent package is particularly where the test unit is generally cylindrical, would comprise a plurality of spaced apart, separately sealed test reagents containing one or more test reagents, the package so designed so as to be penetrated, punctured or dispersed by one end of the probe means on longitudinal movement, so as to provide for contact between the contents of the package and the test reagent. Typically, the probe means penetrates a puncturable or rupturable membrane, which is placed on at least one side, and typically on opposing sides of a generally cylindrical package, or in fact where a tablet is used, is designed to break up a powdered tablet, in contact with the liquid solution and the test sample.

Generally, the sealed reagent package would comprise a plurality of generally separate, individual packages with one or more test reagents having puncturable sealed membranes and opposite radial sides thereof, all selected to be punctured at selected identification poasitions by the probe positions, to provide for adequate contact between the test sample at the end of the probe means and each of the reagents, so that the entire mixture or content thereof, would end up in the reagent.

A numbered type material concentration and form of the

test reagents in each package or alone may widely vary. For example, the test reagents may contain a dried microorganism or other microorganisms, growth and enhancing indicators, such as detergents, ethylene diamine, tetra acetic acid, enhancing reagents to enhance the test results, such as pH or dye color indicators, buffer solutions, saline solutions, water solutions, enzymes, material which bioluminesces, such as luciferin alone and in combination with a luciferin derivative or with other materials which provide bioluminination, as well as low level radioactive isotopes, for example a beta-lactam test, stabilizers, antioxidants, phosphatates and phosphatase substrates, various biological buffers, material such as a chromogen which acts in the presence of an enzyme and a wide variety of other materials.

The invention comprises a method for the detection of a test sample form or on a material, which method includes providing a test apparatus with a sample and test units. Collecting a test sample by use of a probe means, which is stored in a sterile chamber within a sample unit, and thereafter using the probe means, for example, containing a test swab with an end thereof, which may be premoistened so as to collect a test sample, thereafter using the probe means within a chamber to puncture one or more test reagent means, so as to provide for contact within a test unit at one end of the test apparatus of the test samples with one or more test reagents, so that the test method can be carried out, and with the probe moved longitudinally between selected probe positions within the test apparatus. The method also includes employing a test unit, either individually o r by the use of instruments, either alone or as an integral part of the test apparatus, to do the test detection. The test results are determined by in a comparative controlled test or by a minimum threshold level test or by reference to prior

standards. any test method may be typically employed in the test apparatus, the selection of a particular test and test reagents briefly know to persons skilled in the art depending on the particular test. For example, some tests include for example a generally hygiene test for the detection of ATP, for the detection of phosphatase, for the detection of beta--lactams, for the detection of coliform and e-coli, for the detection of organic phosphatides and sulfur drugs. Many other tests, such as for example, those tests shown and described in Charm patents.

The apparatus is composed of two units, the sample unit and the test unit. In one embodiment, the test unit is an integral part of the apparatus and does not need to be removed for final reading of results. Instead, the whole apparatus is inserted to the luminometer for reading. In another embodiment, which call for removal of the test tube unit for analysis, is used because of the portable luminometer constraints. The analyzer, e.g. luminometer, can accommodate the whole apparatus, and therefore better and more simply contain all chemicals in the apparatus for disposal. The cover/chamber sliding mechanism can be controlled after testing by a spiral or raised portion on the plastic in order to control the speed of or stop the downward motion of the probe, and control the timing for each chemical reaction. Some tests will require the use of a timer to allow the full reaction to take place on a timed basis.

The sample unit contains a sterile chamber housing the probe, made of disposable plastic and composed of a chamber cover that holds the probe and the chamber for the probe, which can be made of metal or other materials. The cover and chamber are sealed prior to use to prevent downward movement of the cover, moving the probe into the chamber. A simple sealing mechanism is used, such as heat shrink plastic or

paper that can be torn by a simple twist, to open the chamber and cover and retrieve the probe for sampling.

The chamber is comprised of a water proof housing to enable the probe material to be maintained moist with the proper solution and ready to use.

The probe may be a swab-type device, made of plastic, wood or metal with the tip made of absorbent material such as cotton, or synthetic material (plastic), or a hollow tube; e.g., a disposable pipette. The tip may be used to obtain a sample by a capillary or vacuum suction, or an affinity probe that can adsorb the analyte by bioaffinity binding; e.g., antibodies or receptors, may also be used.

The unit may also contain instructions and a control mechanism, by which the probe, after the sampling step, is inserted into the testing unit and longitudinally moved to puncture the membranes and allow penetration each reagent container.

In an optional embodiment, a squeezing mechanism may be desired for full recovery of the sample and products of the interaction of the sample and the reagents. In this embodiment, the chamber's opening is narrowed to enable the swab, when withdrawn to the non-use position, to squeeze out all liquids into the test microtube for best recovery of color/luminescence products.

The test unit is essentially a transparent test tube (plastic or glass) that contains the active components of a selected test with the test sample. Each chemical is contained within a small cylinder; e.g. a reagent chamber, and inserted in the housing and both top and bottom are sealed with a water- and chemical-resistant membrane made of aluminum foil, plastic or waxed paper, or a combination of the above.

The membrane is thin enough to be fractured, burst, or

punctured by the probe with a slight pressure by the user. The reagents are packaged in the reagent chamber in liquid, dried powder or tablet forms. The number of reagents may vary as required for each test method selected; for example, from one to five ingredients, depending on the test requirements.

Optionally, indicator dye is included with the early reagent (e.g. in reagent A), the first penetrable reagent. This helps to verify that all the chemical interactions during the test are working properly. When the dye is visible in the test housing, it is an indicator of a used device.

The test apparatus system and method will be described for the purposes of illustration only in connection with a series of illustrative test method employing various test apparatus. However, it is recognized that those persons skilled in the art may make various modifications, changes, additions, and improvements to the test apparatus, system and methods without departing form the spirit and scope of the invention.

Brief Description of the Drawings

Fig. 1 is an elevational view of the test wand apparatus of the invention.

Fig. 2 is a sectional view along line 2-2 of the apparatus of Fig. 1.

Fig. 3 is an elevational view of the apparatus of Fig. 1 with the plunger removed.

Fig. 4 is an elevational view of the apparatus of Fig. 1 with the microtube removed and capped.

Fig. 5, with schematic illustrations 5A - G, shows the steps of the test method employing the apparatus of Fig. 1.

Fig. 6 is an enlarged, fragmented, sectional view of the

lower section of the apparatus of Fig. 1 in the non-use position.

Fig. 7 is an enlarged, exploded, fragmented view of the microtube and reagent packages of the apparatus of Fig. 1.

Fig. 8 is a sectional view of another embodiment of a threadable test wand apparatus of the invention.

Description of the Embodiments

Fig. 1 shows the test wand apparatus of the invention 10, with plunger 12 being secured around and outside of elongated cylinder 14. The microtube 16 is attached to the bottom end of the cylinder 14, the microtube 16 having indentations 26 and finger grips 24 to enable a user to manually grasp and remove the microtube 16 from the cylinder 14. A swab 18 is inserted into the interior top end 15 of the plunger and removably secured therein. A foil seal 20 is positioned on the microtube 16 and is removably adhered by self-adhesive backing to the microtube 16. Indicator lines 22 are shown on the top end of the cylinder 14. The bottom end of the plunger 12 and the top end of the cylinder 14 are secured together with a plastic seal 17, and removably secured around the periphery of the plunger 12 and cylinder 14.

In the sectional diagram of Fig. 2, the apparatus of the invention 10 is shown with the plunger 12 having the swab 18 inserted into the interior of top end rod 15. The top of the cylinder 28 is shown with an angular, elliptical cut 19 thereon. Fig. 2 further shows the microtube 16 with inner containment system 49 having units 30 and 32 and space at the bottom 34, the units containing Reagent A 36, Reagent B 38, and Tablet C 40 respectively.

Fig. 3 shows the apparatus 10 with the plunger 12 removed from the cylinder 14, the microtube 16 still being

attached to the end of the cylinder. Fig. 4 shows the apparatus 10 with the microtube 16 detached from the cylinder 14 and sealed with the foil seal 20.

Fig. 5 shows the apparatus of the invention 10 in use, with Fig. 5A showing the apparatus 10 prior to use, with plunger 12, cylinder 14 and microtube 16 attached. Cylinder 14 and plunger 12 are attached with plastic package seal 17. Fig. 5B shows the plunger 12 withdrawn from the cylinder 14, with the swab 18 obtaining a test sample from surface area 48. Fig. 5C shows the plunger 12 being reinserted into the cylinder 14, and being depressed to the first indicator mark 22. Fig. 5D shows the plunger 12 being further depressed into the cylinder 14 at the second indicator mark 22. Fig. 5E illustrates the plunger 12 being depressed fully within the cylinder 14 to moisten the tablet at the bottom of the microtube 16. Fig. 5F shows the microtube 16 after removal from the cylinder 14, with the foil seal 20 being sealed over the microtube 16. Fig. 5G shows the microtube of Fig. 5F being inserted into a luminometer 44 and counted with a counter 46 for testing of the sample.

Fig. 6 shows in further detail the bottom end of the apparatus 10 with the microtube 16. The swab 18, premoistened with swabbing solution, is moving longitudinally and downwardly toward the first prepackaged containment unit 30 with a microbial lysis solution and ATP stabilizer. The second prepackaged containment unit 32 is shown with the buffer optimized for luciferin-luciferase reaction, and the luciferin-luciferase Reagent tablet 34 is shown in the bottom of the microtube 16.

Fig. 7 shows in further detail the single use sequential unit dose containment system 49, with plastic cylinders 30 and 32 containing Reagent A 36 and Reagent B 38. Tablet 42 is shown in position below the units. The system 49 is shown

prior to insertion into the microtube 16. While in the preferred embodiment for the detection of ATP, the above-mentioned reagents are utilized, it is recognized that other combinations of reagents and detection products may be used for specific alternate applications of the test wand apparatus.

Fig. 8 shows another embodiment of the test wand apparatus of the invention 50, with plunger 52 having a rounded top end and threads 56 on the interior surface of the open bottom end. These threads 56 are snug fit to the threads 58 on the outside of top open end of the cylinder 54. A swab 70 is removably inserted into the plunger 52. This embodiment also has a microtube 60 removably secured to the cylinder 54 with a peripheral indentation 66 and finger grip 64 to enable the user to detach the microtube 60 from the cylinder 54. A plastic seal 72 secures the cylinder 54 and plunger 52, and a foil seal 62 is removably secured to the microtube 60. The foil seal 62 is used to cover the microtube 60 in a secure fashion after it is detached from the cylinder for testing. Indicator lines 68 allow the user to control the turning of the plunger with the threads to enable the swab to be longitudinally downwardly inserted into the prepackaged reagent containment system.

In use, the test is performed by removing the plastic seal 17 securing the plunger 12 to the cylinder 14, and removing the plunger 12, which plunger has a premoistened swab 18 removably secured into the interior of the rod 15. After swabbing/sampling the affected area being tested, the plunger 12 and premoistened swab 18 with the sample are re-inserted into the cylinder 14. The cylinder 14 has three indicator markings 22 on the outside. When the plunger 12 with swab 18 is re-inserted into the cylinder 14, it is depressed to the second mark, and the plunger is twirled

twice, breaking into the first containment unit 30 with Reagent A 36. The plunger 12 is then depressed to the third mark and twirled twice more, breaking into the second containment unit 32 with Reagent B 38. The plunger is then depressed fully, breaking into the bottom chamber 34 with Reagent tablet C and is then twirled, moistening the reagent tablet C 42 at the bottom of the microtube 16. The plunger 12 with swab 18, having all three reagents thereon and mixed with the sample on the swab, is withdrawn upwardly and longitudinally into the cylinder 14. The microtube 16 is detached from the cylinder 14 at break point 26 by means of the finger grips 24. After removing the adhesive-backed aluminum foil seal 20, the microtube 16 is then covered with the adhesive cap 20 and counted, such as by a luminometer 44 (see Fig. 5).

After testing, the entire apparatus 10 may be easily disposed of. Further, before use, the entire test wand apparatus 10 may be easily carried and stored in the user's pocket or a portable, lightweight carrying case. The unique single use sequential unit dose containment system 49 within the microtube 16 allows for easy storage and portability, without mixing of the regent chemicals and possible spoilage of the chemicals thereof. The following examples are provided to illustrate optional uses of the sample and test kit apparatus and method:

EXAMPLE 1

Total Hygienic Test - total sanitation ATP monitoring test kit: Pocket Swab, (a trademark of Charm Sciences, Inc., Malden, Massachusetts). The swab contains water or cleaning solution (e.g. detergent such as an anionic like sodium lauryl sulfate, a non-ionic like Triton X-100, a quaternary ammonium like benzalkonium chloride at 0.01-0.3%, for swabbing biofilm and dried microbial film.

The chamber's ingredients are Buffer A: (0.1-0.3 ml) buffer containing phosphoric acid 0.05% and anionic detergents (0.1%) for rapid release of ATP from microorganisms. The buffers could be acids: e.g., trichloroacetic acid or phosphoric acid at 0.01-0.5%, pH 1-3 (e.g. 0.1% phosphoric acid pH 2 and 0.5% Triton X-100), or neutral to alkaline pH buffers such as tris, tricine or carbonate. Detergents can be anionic (sodium lauryl sulfate), neutral (Triton X-100) or cationic (like quaternary ammonium).

The indicator dye: pH indicator such as phenol red (PR) or bromocresol purple (BCP) at 0.0001-0.001%, just enough to be visible to the naked eye. The BCP is yellow in Buffer A, it changes to blue in Step 2 when B and A are mixed, and remains blue in Step 3 when A and B are mixed with Reagent C.

Buffer B is comprised of a neutralizer buffer to optimize the luciferin-luciferase reaction, e.g. 0.05-0.2M of tris, tricine or other biological buffers. Optionally, it is possible to combine Buffer A with Buffer B.

Tablet C contains luciferase and luciferin substrate, e.g. 6-octyl luciferin ester, for detection of ATP. These ingredients are stabilized in a tablet format (see U.S. patent --- and ---, incorporated herein by reference).

EXAMPLE OF RESULTS ENCLOSED AS APPENDIX 1: Sanitation results (RLU) vs. the presence of various microorganism on surfaces in a processing food plant.

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APP. PAGE 23

APPENDIX 1

Table Example of results e PocketSwab in processing food plant

SPC - standard plate count for total aerobic bacteria
CFU - colony forming unit
COLI - coliforms bacteria
ATP - adeninenucleotide triphosphate
RLU - relative light unit

LOCATIO #	Sanitation level	PocketSw ATP (RLU)	SPC CFU	YEAST CFU	MOLD CFU	COLI CFU	Total microbia CFU
1	Good	0	0			0	0
2	Good	0	140	6	0	0	146
3	Good	0	0	0	0	0	0
4	Good	0	0	0	4	0	4
5	Good	0	0	0	0	0	0
6	Good	0	0	0	0	0	0
7	Good	0	0	0	0	0	0
8	Good	0	0	0	0	0	0
9	Good	0	0	0	0	0	0
10	Good	0	20	2	3	0	25
11	Good	0	0	0	0	0	0
12	Good	0	0	0	0	0	0
13	Good	0	0	0	0	0	0
14	Good	0	0	0	0	0	0
15	Good	0	0	0	0	0	0
16	Good	0	0	0	0	0	0
17	Good	0	0	0	0	0	0
18	Good	0	0	0	0	0	0
19	Good	0	10	0	0	0	10
20	Good	0	0	0	0	0	0
21	Good	0	0	0	0	0	0
22	Good	0	10	0	0	0	10
23	low	594	50	0	0	0	50
24	low	647	10	16	4	0	30
25	low	1347	210	8	0	0	218
26	low	2292	110	0	0	10	120
27	low	2437	388	0	0	0	388
28	low	2969	100	0	0	0	100
29	low	3267	2440	23	1	0	2464
30	low	3959	0	0	0	0	0
31	low	3989	0	0	0	280	280
32	low	4460	0	0	0	975	975
33	med	4889	13000	5	0	24	13029
34	med	6697	30	15	0	0	45
35	med	6975	13000	0	0	26	13026
36	med	7174	580	8	32	36	656
37	med	7275	10	123	10	0	143
38	med	8075	460	101	72	0	633
39	med	10625	190	0	52	0	242
40	med	10972	180	2	4	0	186
41	med	15830	300	187	2	0	489
42	med	28067	30	9	164	32	235
43	med	32009	3900	0	0	2	3902
44	med	42685	112	0	3	0	115
45	high	53712	6500	650	455	17	7622
46	high	59019	19500	0	1300	0	20800
47	high	130837	16250	520	178	46	16994
48	high	175154	19500	0	6500	0	26000

EXAMPLE 2

Testing residual raw milk/meat/fish. This test measures the activity of phosphatase as indicative of raw tissue, milk or serum in cooked produce (e.g. pasteurized milk, cooked meat salami, cold cuts, smoked fish). It also can be used to detect cross contamination from raw material in processing surfaces and equipment intended for finishing products.

Commercial name - CHEF Test (a trademark of Charm Sciences, Inc., of Malden, Massachusetts). ALK Test, Cross-contamination test.

The swab contains dry milk or is moistened with water/buffer for meat products and solid dairy products, like cheese.

The chamber ingredients include in Chamber A, a water or saline buffer, pH 6-10 with preservatives (e.g. benzoic acid, sorbate), and a pH indicator such as ~~Phenol~~ ~~Red~~ at 0.001%.

The second chamber contains tablet MP, with Tropix phosphatase substrate (CPD, a product of Tropix, Mass.), freeze dried and made into a tablet.

Chamber 3 contains a stopping solution (0.0025-0.025M EDTA, 0.05-0.2M Tris base or other biological buffers, 0.1-0.3 NaCl, pH 8-11).

EXAMPLE OF RESULTS AS ENCLOSED IN APPENDIX 2: Study of CHEF Test Performance in testing cooked ground beef hamburgers.

APPENDIX 2

Study of CHEF Test Performance
in Heat Processing of Ground Beef

Purpose: To demonstrate the CHEF (Cooking Heat Efficiency) Test's performance, precision and accuracy in predicting doneness of cooked ground beef. Inadequate cooking has been the major cause of stomach poisoning from pathogenic bacteria like E.coli and salmonella.

Introduction: The CHEF Test uses the presence of phosphatase activity to determine whether cooked meats have met CFR (Code of Federal Regulations reference 1) specified cooking temperatures. Acid Phosphatase as an indicator for cooking has been reported in previous literature (see references 2, 3, and 4 below).

Principle: The CHEF Test uses a chemiluminescent substrate for rapid determination of phosphatase activity. the procedure includes the sampling step, which includes using a wet swab to sample the core of the meat (after splitting the meat sample to expose the inner core). Also,m it can be used to swab an equipment surface (e.g., a slicing machine), or other surfaces to test for residual raw meat/milk. In the incubation step, the swab is brought into contact with the chemiluminescent substrate, e.g., CSPD,m a Tropix product, for one to ten minutes at a temperature range from room temperature to 65°C, for example, 55°C for one minute. At the reading step, the reaction is terminated and stabilized by adding a stopping solution and immediately counting relative light units using a luminometer.

Results: The average CHEF Test for raw beef is in the range of 15,000 to 20,000 RLU, while fully cooked beef gives

results in the range of 0-300 RLU (see Table 3).
Results for ground beef heated to various temperatures and hold times are listed in Table 2.

Discussion: Using the results for fully cooked meat, a cut off for determining incompletely cooked meat can be set at the upper range (e.g. 300 RLU). In our field samples (Table 3) all the hamburgers were properly cooked (all results below 300 RLU). In our own cooking experiment, (Table 2), we effectively screen low temperature cooked product (Samples 1-4) from adequately processed and cooked products (sample 5 and 6).

Conclusion: The CHEF Test accurately detects raw meat and also can distinguish fully cooked meats from incompletely cooked meats. Meat processed at a temperature 2°C below CFR specifications and for thirty seconds too short a time (sample 4), was identified as positive in this study. Samples properly processed, and hamburgers purchased from a local restaurant, were negative for residual raw meat.

Table 2. CHEF Test results (RLU) of various ground beef samples held at various temperatures and times.

	Sample #1	Sample #2	Sample #3	Sample #4	Sample #5	Sample #6
Temp. °C (°F):	53 (128)	57 (135)	59 (138)	63 (145)	65 (149)	69 (156)
Hold Time ->:	60 sec.	60 sec.	60 sec.	60 sec.	60 sec.	16 sec.
Replicate #	RLU	RLU	RLU	RLU	RLU	RLU
#1	10536	12490	11622	2795	10	123
#2	17784	22940	5481	3903	0	0
#3	14325	8411	5040	2113	0	0
#4	11979	6309	17881	2060	0	0
#5	21310	12832	10475	4969	186	0
#6	21426	6264	11022	5766	188	227
Average	16227	11541	10254	3601	64	58
+/- Range	4676	6285	4704	1542	95	96
% activity	95	68	60	21	3.8	3.4

A dozen hamburgers purchased at a local food chain tested on the CHEF Test are reported in Table 3.

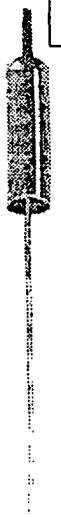
Table 3. CHEF Test (RLU) of Hamburgers from Restaurant

Hamburger #	CHEF (RLU)	Hamburger #	CHEF (RLU)
1	0	7	37
2	0	8	0
3	0	9	0
4	0	10	0
5	0	11	0
6	0	12	0

CHEF Test™ Procedure

CHEF-A01
9/15/95

APPENDIX 2



1

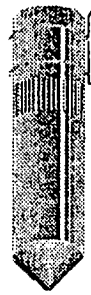
Withdraw plunger.
Slice sample.
Swab center of cooked product.

2

Reinsert plunger.
Depress to mark 2.

3

Depress to mark 3, twirl to disperse tablet.
Wait three minutes.



4

Depress to final mark.
Withdraw to mark 1.
Detach microtube and count.

CHARM SCIENCES INC.
36 FRANKLIN STREET MALDEN MA 02148 USA
800 343-2170 FAX 617 322-3141
Nothing works like a Charm.

EXAMPLE 3

Chemical and antibiotic residue test - Testing of residual antibiotics in milk, urine, and meats.

The swab is dry for sampling of water, mile, meat serum or urine.

The chamber ingredients are comprised of water or 0.005-0.1 phosphate buffer pH 5-8 in Chamber One. In Chamber Two, the tablet contains dried microorganisms, such as naturally luminescent bacteria such as P. phosphoreum, (Canadian Pat #1103050) or, genetically modified bacteria (e.g. E.coli mutant used in the Toxi-Chromotest EBPI, Ontario, Canada); a growth and maintenance nutrient (see patent # 5,354,663 incorporated herein by reference), and a growth or activity indicator like chromogen, which in the presence of an enzyme, such as D-galactosidase or phosphatase, can produce color or luminescence (e.g., Tropix luminescence substrates: CSPD, Galacton-Plus).

Chamber Three contains an enhancing reagent, such as fluorescamine or Tropix enhancing reagents (Emerald, Sapphire).

The procedure for this test comprises obtaining a test sample with the probe means, inserting the swab into the buffer compartment, inserting the swab into the buffer compartment, inserting it into the tablet compartment, and the inserting the swab into the chromogen. It should be noted that the tablet and chromogen can be contained in a single compartment.

The test samples should be incubated for 1-120 minutes and the luminescence then recorded. Inhibition of luminescence indicates the presence of a chemical inhibitor in the sample. For example, using E.coli and Tropix

Galacton-plus substrate in E*Colite/ColiGel media (a trademark of Charm Sciences, Inc., of Malden, Massachusetts), can be used to detect antibiotics such as quinolones, and others. Using B-st, (see #patent #5,354,663) a variety of antibiotics can be detected in about 60-120 minutes using color change or change in luminescence substrate.

///// Each test kit is fully packaged all in one device, including the reagents, which greatly simplifies the test, making it user-friendly. The test utilizes simple steps which are controlled by the plunger and indicator marks, and has rupturable seals, such as aluminum foil seals, that separate the various compartments. It eliminates the need to prepare reagents, and no pipettes or dispensers are needed. This device eliminates operational mistakes due to inaccurate pipettes. Since all the reagents, liquid and tablets, are individually packaged in plastic cylinders which are sealed with aluminum foil, under optimum conditions, the test kit has excellent shelf life stability, with an expectation of over two month's stability at room temperature. The test device can be easily carried and used in any place, for example, in a processing plant, without restrictions.

The invention will be described for the purposes of illustration only in connection with the monitoring of ATP; however, it is recognized that various modifications, changes, improvements and additions may be made to the illustrated embodiment by those persons skilled in the art without departing from the spirit and scope of the invention.

Thus, the test wand apparatus of the invention provides for a safe, convenient, lightweight and inexpensive test apparatus that may be stored for longer periods and easily transported for use. further, the invention is easy, neat and convenient to use. The prepackaged single use sequential unit dose containment system allows for fewer user errors in

preparing reagent chemicals for use. While the single use packaging system of the invention is shown and described herein for the testing of ATP for sanitation purposes, it is recognized that the apparatus, system and method may be used for a wide variety of product applications.

What is claimed is:

Claims

Claim 1. A test apparatus having a sample unit and a test unit for the detection of a test sample from or on a material, which apparatus comprises:

a) a sample unit having:

i) a probe means having a first and second end, with said first end to obtain a test sample in use to be collected from or on a material;

ii) a sterile chamber having a first and second end and adapted to receive and retain therein prior to use and after use said probe means and having a cover for said first end of said chamber;

iii) means to retain said probe means within said chamber prior to use;

iv) probe position means comprising a plurality of selected identification positions between said probe means and said chamber to identify the position of said probe means within said chamber; and

v) means to move longitudinally said first end of said probe means within said chamber for use to selected identification positions; and

b) a test unit attached to the sample unit having;

i) a reagent housing with a bottom and having a first and second end, said first end attached to said second end of said chamber, said housing adapted for use with or in a test instrument for the test sample; and

ii) test sample reagent means to contact the test sample and comprising at least one sealed reagent package, characterized by a puncturable membrane adapted to be penetrated by the longitudinal movement of said first end of the probe means after use, and with the test sample at a selected identification position; the test sample and the

reagent means in combination providing a selected test for the detection of the test sample when the test sample and the reagent means are combined in the test unit.

Claim 2. The apparatus of claim 1 wherein said test unit and said sample unit are comprised of disposable transparent plastic tube material.

Claim 3. The apparatus of claim 1 wherein said probe means comprises an elongated element secured at said second end to said cover and having a test sample collection material at said first end.

Claim 4. The apparatus of claim 3 wherein said first end of said probe means is liquid-moistened to aid in collection of the test sample.

Claim 5. The apparatus of claim 1 wherein the means to retain said probe means comprises a user-removable tape means.

Claim 6. The apparatus of claim 5 wherein said tape means comprises a heat-shrunk material.

Claim 7. The apparatus of claim 1 wherein the probe position means comprises a plurality of selected spaced-apart marks on the chamber.

Claim 8. The apparatus of claim 1 wherein the means to move comprises the cover and the chamber in a close, snug-fitting slidable arrangement after removal of the means to retain.

Claim 9. The apparatus of claim 1 wherein the means to move comprises spiral means to provide for the spiral longitudinal movement by a user to the selected identification positions.

Claim 10. The apparatus of claim 1 wherein the probe position means comprises selected, spaced-apart, user-marked identification positions on said chamber to include:

a) a nonactive position for said first end of said probe

means prior to and after use;

b) a position of said first end of the probe means to puncture the membrane of at least one of the reagent means; and

c) a position wherein said first end of the probe means, with the test sample and one or more reagents means is positioned to deposit the test sample and punctured reagent means in the reagent housing.

Claim 1-. The apparatus of claim 1 wherein the cover fits over said first end of the chamber and said second end is open, and wherein the probe means comprises an elongated element secured generally centrally to the inside of the cover and contains a probe means of fibrous material at the other end of the probe means.

Claim 11. The apparatus of claim 1 wherein the test unit with the test sample and test reagent means therein may be removed from the sample unit for testing.

Claim 12. The apparatus of claim 11 wherein the apparatus is characterized by a mechanically weakened section between the sample and test unit which permits detaching of the test unit from the sample unit by a user.

Claim 13. The apparatus of claim 11 wherein the apparatus includes a means for capping the open detached end of the test unit after removal.

Claim 14. The apparatus of claim 13 wherein the means for capping comprises an adhesive foil cap, which foil cap is temporarily and removably placed on the exterior of the test unit for use by the user.

Claim 1-. The apparatus of claim -- wherein the sample unit and test unit are longitudinally but detachedly secured together.

Claim 1-. The apparatus for claim 1- wherein a section between the sample unit and the test unit is radially

narrowed.

Claim 15. The apparatus of claim 1 wherein the test reagent means comprises a plurality of sealed packages composed of test reagents which tend to prereact or to be less effective with time if directly admixed with the test sample.

Claim 16. The apparatus of claim 1 wherein the test reagent means comprises a plurality of sealed, longitudinally aligned separate packages of test reagents in generally cylindrical form and having opposing probe means-puncturable membranes.

Claim 17. The apparatus of claim 1 wherein the test reagent means with the test sample provides for a visual detection test for the test sample in the test unit.

Claim 18. The apparatus of claim 1 wherein the test reagent means comprises a plurality of test reagents having at least one buffer test reagent with an indicator dye therein and at least one test reagent for detection of the test sample in the presence of the buffer and dye indicator.

Claim 19. The apparatus of claim 1 for the detection of ATP from a material or surface and wherein said first end of the probe means is pre-moistened and the reagent means comprises a plurality of test reagents to include:

a) a buffer solution with a detergent and an indicator dye, with optionally a neutralizing buffer solution; and

b) a tablet which comprises luciferase and a luciferin substrate for the detection of ATP in the sample.

Claim --. The apparatus of claim -- wherein the luciferin derivative is a C ester luciferin acetate.

Claim 20. In combination, the test apparatus of claim 1 and a luminometer as a test instrument to measure the chemiluminescence in the test unit for the detection of the test sample.

Claim 21. The combination of claim 20 wherein the test unit is not detached and the test unit of the apparatus is placed in a luminometer measuring section for detection.

Claim 22. The combination of claim 20 wherein the test unit is detached from the sample unit, means to cap applied, and only the luminescence of the detached test unit is measured by the luminometer.

Claim 23. The apparatus of claim 1 for the detection of phosphatase from a material or surface and wherein the one end of the probe means is pre-moistened and the test reagent means comprises a plurality of test reagents to include:

a) a water or saline buffer solution;

b) a tablet which comprises a chemiluminescent phosphatase substrate to measure the phosphatase on the test sample; and

c) a biological buffer solution to stop the reaction of the phosphatase with the test sample.

Claim 24. The apparatus of claim 1 for the detection of chemical or antibiotic test samples and wherein the test reagent means comprises a plurality of test reagents to include:

a) water or a buffer solution;

b) a tablet which comprises a microorganism, a nutrient for the microorganism and a growth-activity indicator; and

c) an enhancing reagent to enhance the test results and permit test measurement for the detection.

Claim 25. In combination, the apparatus of claim 24 with a means to incubate the test unit with the test sample and test reagents.

Claim 26. The apparatus of claim 1 wherein the chamber or housing comprises an elongated, flexible, transparent, plastic tube material with the cover fitted over at one end of the chamber, to permit the user to hand squeeze together

the apparatus at a position between the ends of the apparatus for substantially full receiving of the test sample and punctured reagents into the reagent housing.

Claim 27. In combination, the apparatus of claim 1 which includes timer means to time the reaction of the test sample and test reagents.

Claim 28. The apparatus of claim 27, wherein the reagent means includes naturally luminescent bacteria, genetically modified bacteria, growth and maintenance nutrient, and a chromogen, which in the presence of an enzyme, produces a detectable color or bioluminescence.

Claim 29. A method for the detection of a test sample from or on a material, in which the test sample is combined with test reagents to provide test results, which method includes:

a) providing an elongated test apparatus with a sample unit constructed and arranged to obtain a test sample to retain a probe means therein, and a test unit to provide test results;

b) removing the probe means and collecting the material to be tested to obtain a test sample on the probe means;

c) inserting the probe means within the test apparatus;

d) longitudinally moving the probe means in the test apparatus, with the test sample, through a series of probe means identification positions of the test apparatus;

e) puncturing one or more packaged test reagents selected for the particularly test method for the sample by the longitudinal movement of the probe means to provide a contacting of the test reagents and the test sample from the probe means in the test unit; and

f) observing the test results in the test unit.

Claim 31. The method of claim -- which includes providing a probe means with an elongated element secured at

a first end to a cover over the sample unit and having a premoistened fibrous probe sample collection material at said first end.

Claim 34. The method of claim 29 which includes employing as the probe position means a plurality of selected spaced-apart marks on the chamber.

Claim 35. The method of claim 29 which includes slidably moving the probe means to the identification position.

Claim 36. The method of claim 29 which includes spirally moving the probe means to the identification position.

Claim 38. The method of claim -- which includes detaching the test unit only with the test sample and test reagents therein from the sample unit for use in a test instrument.

Claim 40. The method of claim 38 which includes sealing said open end of the detached test unit with a cap means after removal of the test unit from the sample unit.

Claim 41. The method of claim 38 which includes providing as the cap means an adhesive foil cap, temporarily and removably adhering said cap means on the exterior of the test unit.

Claim 42. The method of claim 38 which includes providing the test reagent with a plurality of separate sealed packages of test reagents which tend to prereact or to be less effective with time if directly admixed and reacted with the test sample.

Claim 43. The method of claim 29 which includes providing a plurality of separately sided longitudinally aligned, sealed packages of test reagents in general cylinder form and having opposing puncturable membrane sides puncturable by the probe means of longitudinal movement for

containing the test reagent means.

Claim 44. The method of claim 29 which includes selecting a visual detection test for the test sample with the test reagent means with test samples in the test unit.

Claim 45. The method of claim 29 which includes providing a plurality of packaged test reagents in the test unit having at least one buffer test reagent, an indicator dye therein and at least one test reagent for reacting with the test sample in the presence of the buffer and dye indicator.

Claim 46. The method of claim 29 which includes providing a test method for the detection of ATP from a material surface and wherein the test reagent comprises a plurality of test reagents to include:

a) a buffer solution with a detergent and an indicator dye;

b) optionally a neutralizing buffer solution; and

c) a tablet which comprises luciferase and a luciferin substrate for the detection of ATP in the sample.

Claim 47. The test method of claim -- which includes measuring the reaction chemiluminescence of the test sample with a luminometer.

Claim 48. The combination of claim 46 which includes placing the test unit of the entire apparatus in a luminometer measuring section.

Claim 50. The method of claim 29 for the detecting of phosphatase from a material, comprising a plurality of test reagents including:

a) a water or saline buffer solution;

b) a tablet which comprises a chemiluminescent phosphatase substrate to measure the phosphatase of the test sample; and

c) a biological buffer solution to stop the reaction of

the phosphatase with the test sample.

Claim 51. The method of claim 29 for the detecting of chemicals and antibiotics from a material, comprising a plurality of test reagents to include:

a) water or a buffer solution;

b) a tablet which comprises a microorganism, a nutrient for the microorganism and a growth or activity indicator; and

c) an enhancing reagent to enhance the test results and permit test measurement.

Claim 52. The method of claim -- which includes timing and incubating the test unit with the test sample and test reagents.

Claim 53. The method of claim 29 which includes the user hand squeezing together in the chamber or housing for substantially full receiving of the test sample and reagents into the test unit.

Abstract of the Disclosure

The invention concerns a test apparatus system and method for the testing of a test sample on or in a material, such as body fluids or food, particularly adapted to a bioluminescent test such as for the detection of ATP or phosphatase or other material. The test apparatus comprises a longitudinally elongated, plastic disposable transparent tube having at one end a sample unit at the other end a test unit which are connected, and which includes a cover with a probe containing a swab at one end. the test apparatus includes probe positioning marks on the chamber of the sample unit, so that the probe may be moved between selected test positions, and as it moves form a non use to a use position, various test reagents which are sealed within the test unit are punctured by the probe, and the test sample and the test units are gathered together in the test unit. The test unit may be detachable for in-field use and then tested in a luminometer, or the entire test apparatus may be then taken and the test unit nondetachable employed to determine the test results. The system may include, as desired, a portable or laboratory luminometer or other test equipment and may include, where desired, a timer and incubation means where timing and incubation is a part of the test method. The test apparatus and system may be employed in a wide variety of tests, wherein one or more test reagents are employed, such as tests for ATP, phosphatase, sulfur drugs, organophosphate residues, ---, beta-lactams and the like.

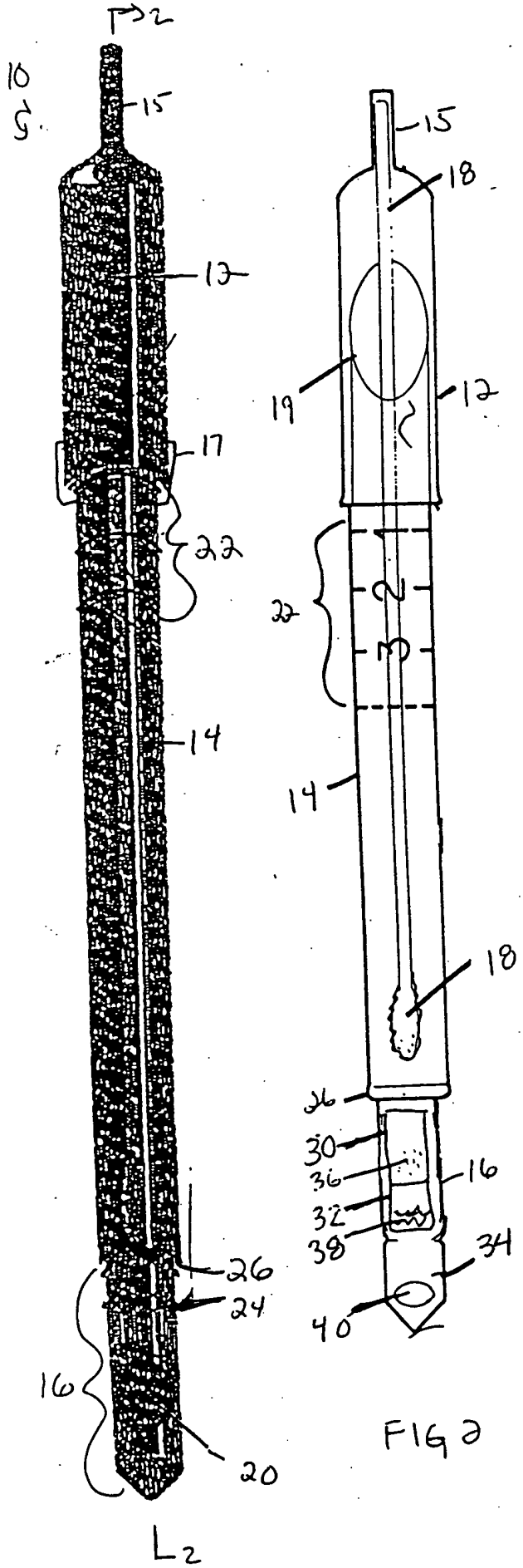


FIG 2

FIG 3

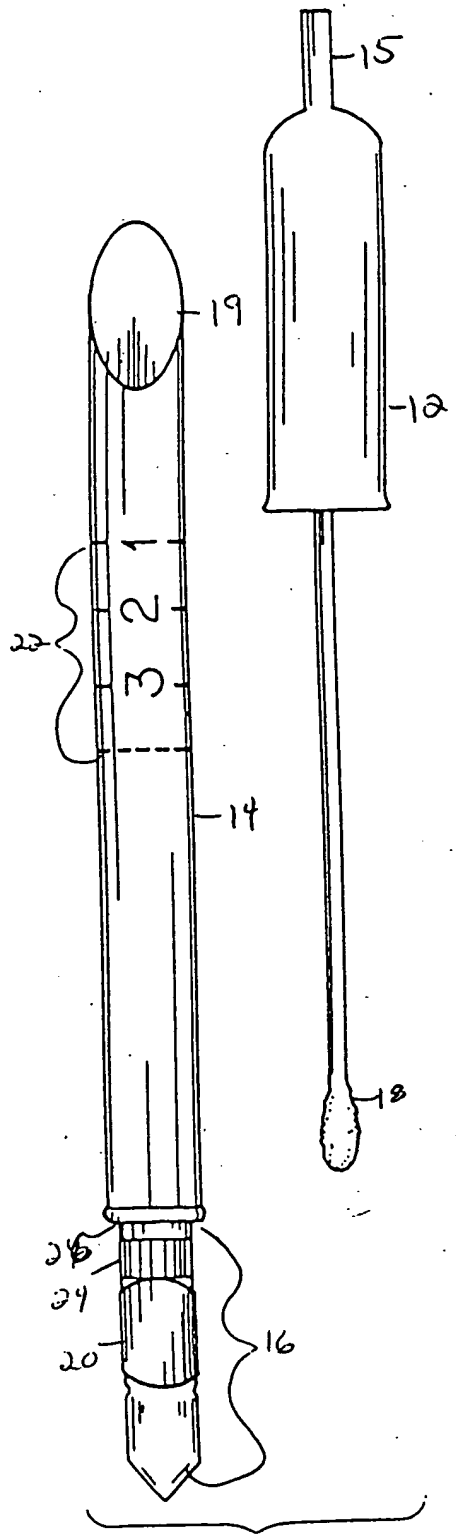
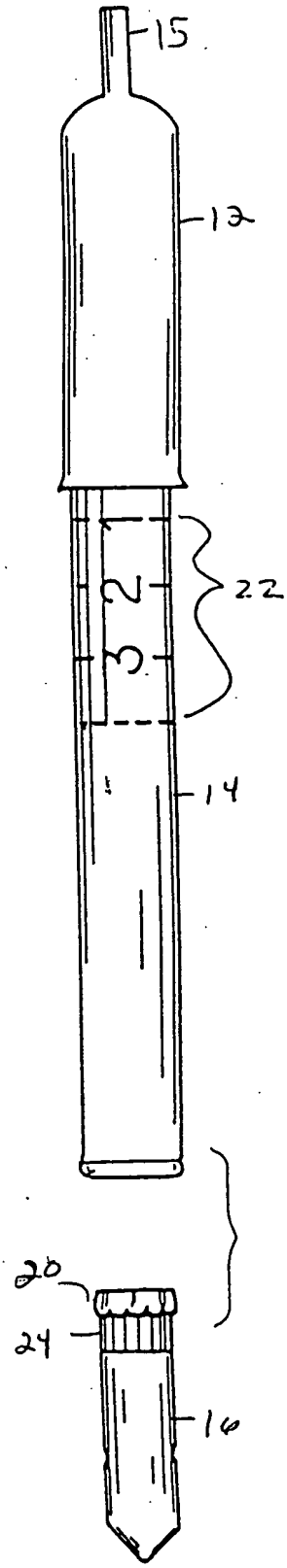


FIG 4



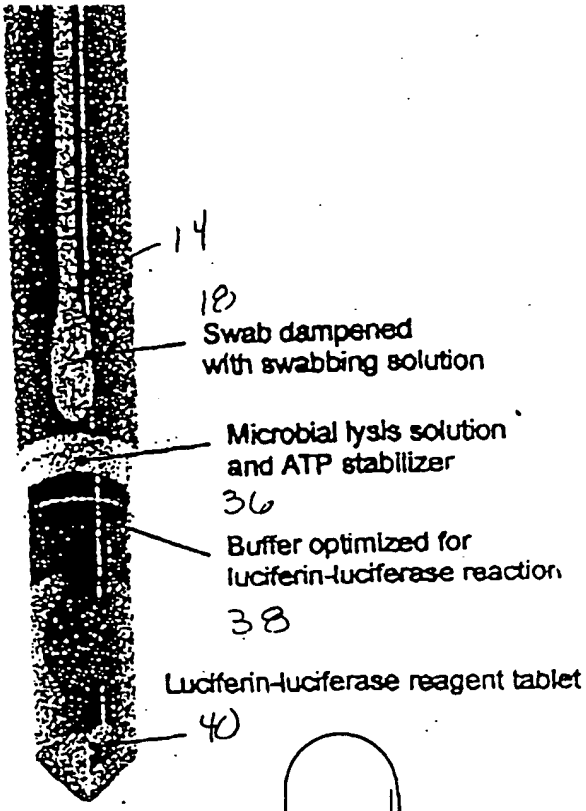

14
18
Swab dampened with swabbing solution
Microbial lysis solution and ATP stabilizer
36
Buffer optimized for luciferin-luciferase reaction
38
Luciferin-luciferase reagent tablet
40

FIG 6

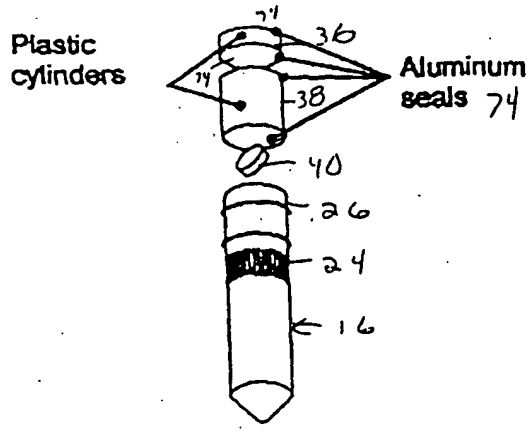

Plastic cylinders
Aluminum seals 74
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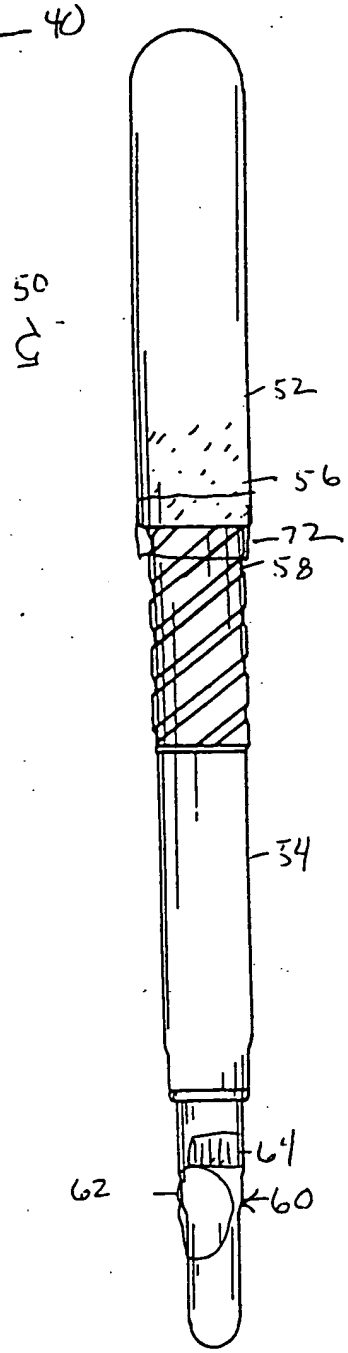
FIG 7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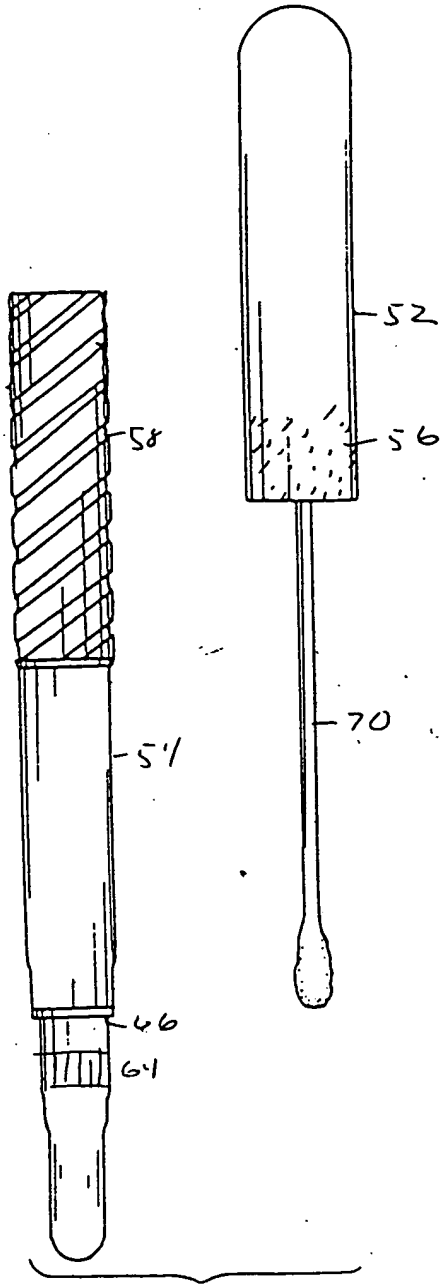
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PTO/SB/58 (07-03)
Approved for use through 7/31/2003. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

REQUEST FOR ACCESS TO AN ABANDONED APPLICATION UNDER 37 CFR 1.14

In re Application of

Application Number	Filed
60/007,585	11/27/95

Bring completed form to:
File Information Unit
Crystal Plaza Three, Room 1D01
2021 South Clark Place
Arlington, VA
Telephone: (703) 308-2733

Paper No. 2

I hereby request access under 37 CFR 1.14(a)(1)(iv) to the application file record of the above-identified ABANDONED application, which is identified in, or to which a benefit is claimed, in the following document (as shown in the attachment):

United States Patent Application Publication No. _____, page, _____ line _____.

United States Patent Number 5827675, column Face, line, _____ or

WIPO Pub. No._____, page _____, line _____.

Related Information about Access to Pending Applications (37 CFR 1.14):

Direct access to pending applications is not available to the public but copies may be available and may be purchased from the Office of Public Records upon payment of the appropriate fee (37 CFR 1.19(b)), as follows:

For published applications that are still pending, a member of the public may obtain a copy of:
the file contents;
the pending application as originally filed; or
any document in the file of the pending application.

For unpublished applications that are still pending:

(1) If the benefit of the pending application is claimed under 35 U.S.C. 119(e), 120, 121, or 365 in another application that has: (a) issued as a U.S. patent, or (b) published as a statutory invention registration, a U.S. patent application publication, or an international patent application publication in accordance with PCT Article 21(2), a member of the public may obtain a copy of:
the file contents;
the pending application as originally filed; or
any document in the file of the pending application.

(2) If the application is incorporated by reference or otherwise identified in a U.S. patent, a statutory invention registration, a U.S. patent application publication, or an international patent application publication in accordance with PCT Article 21(2), a member of the public may obtain a copy of:
the pending application as originally filed.

Michael D. Linton
Signature

9/4/03
Date

Michael D. Linton
Typed or printed name

_____
Registration Number, if applicable

703-553-0000 #255
Telephone Number

FOR PTO USE ONLY

Approved by: _____
(initials)

Unit: _____

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

1ST EXAMINER 208	DATE 2/7
2ND EXAMINER	DATE

APPLICATION NUMBER	TYPE APPL	FILING DATE MONTH DAY YEAR	SPECIAL HANDLING	GROUP ART UNIT	CLASS	SHEETS OF DRAWING
60/007585	9	11 27 95	0			1 / 2

TOTAL CLAIMS	INDEPENDENT CLAIMS	SMALL ENTITY?	FILING FEE	FOREIGN LICENSE	ATTORNEY DOCKET NUMBER
			150	Y	95-712

CONTINUITY DATA

STATUS CODE	PARENT APPLICATION SERIAL NUMBER	PCT APPLICATION SERIAL NUMBER	PARENT PATENT NUMBER	PARENT FILING DATE MONTH DAY
		PCT/		
		PCT/		
		PCT/		
		PCT/		
		PCT/		

PCT/FOREIGN APPLICATION DATA

FOREIGN PRIORITY CLAIMED	COUNTRY CODE	PCT/FOREIGN APPLICATION SERIAL NUMBER	FOREIGN FILING DATE MONTH DAY YEAR

01/007585

CONTENTS

Date Entered or Counted		Date Received or Mailed
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	2. Request For Access	9-4-03
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/014,154	12/06/2001	Richard T. Skiffington	0656-008US6	1022

32665 7590 03/23/2006

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EXAMINER	
BEISNER, WILLIAM H	

ART UNIT	PAPER NUMBER
1744	

DATE MAILED: 03/23/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

DOCKETED
Action Due N/A
Docket Date —
Initials LM1 4/6/06

PTO-90C (Rev. 10/03)

Interview Summary	Application No.	Applicant(s)	
	10/014,154	SKIFFINGTON ET AL.	
	Examiner	Art Unit	
	William H. Beisner	1744	

All participants (applicant, applicant's representative, PTO personnel):

(1) *William H. Beisner*. (3)____.

(2) *Leslie Meyer-Leon*. (4)____.

Date of Interview: *16 March 2006*.

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☒ No.
If Yes, brief description: ____.

Claim(s) discussed: *All pending claims*.

Identification of prior art discussed: *NONE*.

Agreement with respect to the claims f)☒ was reached. g)☐ was not reached. h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *See Continuation Sheet*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

WILLIAM H. BEISNER
PRIMARY EXAMINER
GROUP 744

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)
In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: With respect to the office action dated 1/31/06, Applicants' representative pointed out that the record did not make it clear that claims 1,2, 4-7, 10, 12, 14, 15, 17-19, 23, 24 and 26 have benefit of the filing date of U.S. Provisional Application No. 60/001,081 filed 12 July 1995. Applicants' representative requested that the Examiner clarify the record by issuing a supplemental action. The Examiner agreed to issue a supplemental action which clarifies that the above claims have benefit of the filing date of provisional application 60/001,081, filed 12 July 1995.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/014,154	12/06/2001	Richard T. Skiffington	0656-008US6	1022

32665 7590 03/23/2006

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CENTERVILLE, MA 02632-1210

EXAMINER	
BEISNER, WILLIAM H	
ART UNIT	**PAPER NUMBER**
1744	

DATE MAILED: 03/23/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 10/03)

DOCKETED
Action Due N/A
Docket Date —
Initials LML 4/5/06

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APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.

EXAMINER	
ART UNIT	PAPER
	20060322

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

In response to applicant's telephone inquiry dated 3/16/06 regarding the last Office action, the following corrective action is taken.

Claims 1, 2, 4-7, 10, 12, 14, 15, 17-19, 23, 24 and 26 have benefit of the filing date of U.S. Provisional Application No. 60/001,081, filed 12 July 1995, as evidenced by the prosecution history (See page 16, paragraph 17, of the Office Action dated 10/4/2004).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to William H. Beisner whose telephone number is 571-272-1269. The examiner can normally be reached on Tues. to Fri. and alt. Mon. from 6:15am to 3:45pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gladys J. Corcoran can be reached on 571-272-1214. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

William H. Beisner
Primary Examiner
Art Unit: 1744